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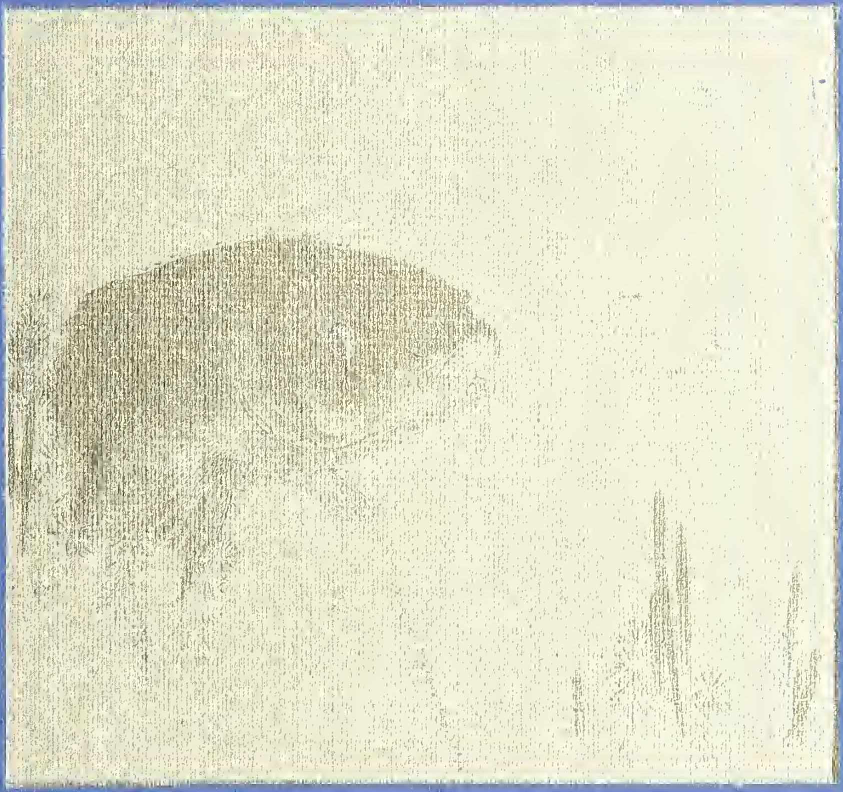

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Use of Small Fish Species in Carcinogenicity Testing

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Use of Small Fish Species in Carcinogenicity Testing

Proceedings
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Bethesda, Maryland

December 8–10, 1981

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National Cancer Institute

Editor:

Karen L. Hoover, Sc.D.

TABLE OF CONTENTS

Page

Foreword

Karen L. Hoover . . . ix

Dedication: An Appreciation of Dr. Mearl F. Stanton

Clyde J. Dawe . . . 1

Session I. Proposed Species for Carcinogenicity Testing In Vivo

Characteristics of a Fish Colony of *Poeciliopsis* and Its Use in Carcinogenicity Studies With 7,12-Dimethylbenz[a]anthracene and Diethylnitrosamine

R. Jack Schultz and Mary E. Schultz . . . 5

Usefulness of the Hermaphroditic Marine Fish, *Rivulus marmoratus*, in Carcinogenicity Testing

Christopher C. Koenig and Michael P. Chasar . . . 15

Usefulness of the Medaka, *Oryzias latipes*, as a Test Animal: DNA Repair Processes in Medaka Exposed to Carcinogens

Takatoshi Ishikawa, Prince Masahito, and Shozo Takayama . . . 35

The Amazon Molly, *Poecilia formosa*, as a Test Animal in Carcinogenicity Studies: Chronic Exposures to Physical Agents

Avril D. Woodhead, Richard B. Setlow, and Virginia Pond . . . 45

Attainment of Minimal Biological Variability and Measurements of Genotoxicity: Production of Homozygous Diploid Zebra Fish

George Streisinger . . . 53

Usefulness of the Guppy, *Poecilia reticulata*, in Carcinogenicity Testing: Special Advantages and Problems

Edmund J. Zimmerer . . . 59

Use of Aquarium Fish, *Danio rerio* and *Poecilia reticulata*, as Test Species for Evaluation of Nitrosamine Carcinogenicity

Veniamin V. Khudoley . . . 65

Carcinogenesis Studies on Guppies

Károly Simon and Károly Lapis . . . 71

Usefulness of *Cyprinodon variegatus* and *Fundulus grandis* in Carcinogenicity Testing: Advantages and Special Problems

Lee A. Courtney and John A. Couch . . . 83

Xiphophorus as an In Vivo Model for Studies on Oncogenes

Fritz Anders, Manfred Schartl, and Angelika Barnekow . . . 97

Use of the Mudminnow, *Umbra* sp., in an In Vivo Sister Chromatid Exchange Test

Andrew D. Kligerman, William E. Bishop, and Laura C. Valentine . . . 111

Hyperplastic Lesions of the Primitive Meninx of Fathead Minnows, *Pimephales promelas*, Induced by Ammonia: Species Potential for Carcinogen Testing

Charlie E. Smith . . . 119

Session II. Alternative Testing Systems With the Use of Fish

Page

Rainbow Trout Embryos: Advantages and Limitations for Carcinogenesis Research

Jerry D. Hendricks, Theodore R. Meyers, John L. Casteel, Joseph E. Nixon, Patricia M. Loveland, and George S. Bailey 129

Correlation of Epizootiological Observations With Experimental Data: Chemical Induction of Chromatophoromas in the Croaker, *Nibea mitsukurii*

Ikuo Kimura, Nobuhiko Taniguchi, Hidemi Kumai, Isao Tomita, Naohide Kinae, Koichi Yoshizaki, Masao Ito, and Takatoshi Ishikawa 139

Preliminary Studies on the Usefulness of Medaka, *Oryzias latipes*, Embryos in Carcinogenicity Testing

James E. Klaunig, Bruce A. Barut, and Peter J. Goldblatt 155

Establishment of Fish Hepatocyte Cultures for Use in In Vitro Carcinogenicity Studies

James E. Klaunig 163

Primary Cell Cultures From the Teleost, *Cyprinodon variegatus:* Culture Establishment and Application in Carcinogen Exposure Studies

Billy J. Martin, Rudolph D. Ellender, Susan A. Hillebert, and Mitchell M. Guess 175

Culture of Embryos and Cells From Platyfish and Swordtails for Carcinogenicity Testing and Studies on Melanoma Formation

Ursula Vielkind and Jürgen R. Vielkind 179

Session III. Maintenance of Fish in the Laboratory

Physiological Responses of Experimental Fish to Stressful Conditions

John B. Gratzek and Robert Reinert 187

Procedures for Use of Freshwater Fishes in the Development of Reproducible Toxicological Information

Leif L. Marking 195

Nutritional Requirements of Experimental Fish

Harry K. Dupree 201

Serological Alterations in Carcinogen-exposed Teleosts: Procedures for Preparation and Analysis of Samples From Small Fish

C. Brent Meador, Robert L. Middlebrooks, and Billy J. Martin 211

Panel Discussion

John B. Gratzek 217

Debate: Mouse Versus Minnow: The Future of Fish in Carcinogenicity Testing

Clyde J. Dawe and John A. Couch 223

Session IV. Special Problems and Topics Related to the Use of Fish in Cancer Research

Effect of Age and Exposure to a Carcinogen on the Structure of the Medaka Liver: A Morphometric Study

David E. Hinton, R. Clark Lantz, and James A. Hampton 239

Pseudoneoplasms in Ectothermic Animals

John C. Harshbarger 251

Page

Hyperplastic Thyroid Lesions in Fish

Karen L. Hoover 275

Morphological Survey of Teleost Organs Important in Carcinogenesis With Attention to Fixation

David E. Hinton, Elizabeth R. Walker, Carlin A. Pinkstaff, and Edwin M. Zuchelkowski 291

Histological Progression of Hepatic Neoplasia in Rainbow Trout (*Salmo gairdneri*)

Jerry D. Hendricks, Theodore R. Meyers, and Dennis W. Shelton 321

Effects of Temperature and Partial Hepatectomy on the Induction of Liver Tumors in *Oryzias latipes*

Yoriko Kyono-Hamaguchi 337

Factors Influencing Methylazoxymethanol Acetate Initiation of Liver Tumors in *Oryzias latipes*: Carcinogen Dosage and Time of Exposure

Kazuko Aoki and Hiromichi Matsudaira 345

Session V. Metabolic Handling of Xenobiotics by Fish

Biotransformation of Chemicals by Fish: An Overview

John J. Lech and Mary Jo Vodicnik 355

Heterogeneity of Hepatic Aryl Hydrocarbon Hydroxylase Activity in Feral Winter Flounder: Relevance to Carcinogenicity Testing

John R. Bend, Gary L. Foureman, Zvi Ben-Zvi, and Phillip W. Albro 359

Patterns of Benzo[a]pyrene Metabolism by Varied Species, Organs, and Developmental Stages of Fish

John J. Stegeman, Bruce R. Woodin, and Robert L. Binder 371

Dietary Modification of Aflatoxin B_1 Carcinogenesis: Mechanism Studies With Isolated Hepatocytes From Rainbow Trout

George S. Bailey, Matthew J. Taylor, Patricia M. Loveland, Janet S. Wilcox, Russell O. Sinnhuber, and Daniel P. Selivonchick 379

Comparative Benzo[a]pyrene Metabolite Patterns in Fish and Rodents

Paul Melius 387

Benzo[a]pyrene Metabolism in Marine Fish and Some Analytical Aspects of Its Metabolites

W. Peter Schoor 391

Summary and Recommendations: A Consensus Report

Consensus Committee 397

Participants 405

FOREWORD[1, 2]

Novel approaches to the investigation of a significant scientific problem, such as the testing of suspect carcinogens, generally suffer from a lack of attention and an abundance of skepticism by the rest of the scientific community. On the other hand, the limited scope and relative youthfulness of the area can make for unique vitality and excitement among its proponents. This attitude was most certainly noted when scientists from Japan, Canada, Germany, and the United States met in Lister Hill Center at the National Institutes of Health, Bethesda, Maryland, in December, 1981, to participate in this Symposium on the use of small fish species in carcinogenicity testing. For 3½ days, 31 researchers in the field met to discuss their experimental results, to address problems unique to the use of these animals in laboratory and field studies, and to propose recommendations for future directions of this research. The formal papers presented in this Monograph cannot reflect adequately the enthusiasm and dedication of these investigators for their research, as was clearly evident at the Symposium, but they do convey the innovation and thoughtfulness of its proponents that accompany the advancement of a young and potentially useful area of investigation. The proposed value of fish in laboratory and field studies chiefly centers around two attributes: 1) Certain small fish species, such as the medaka, may develop chemically induced tumors within a remarkably short period (as little as 5 wk), and 2) fish spend 24 hours of every day within the water, and, more to the point, within and along the nation's often chemically contaminated waterways and shorelines. Such characteristics argue for the possible use of fish as subjects for short-term in vivo screening of suspect carcinogens in a multitiered testing scheme and as monitors of environmental pollution in field surveys of the water. Although this Symposium was entitled "Use of Small Fish Species in Carcinogenicity Testing," it was essential that the role of larger fish species (particularly the trout) be discussed in light of the excellent laboratory studies conducted with rainbow trout by scientists at Oregon State University and the numerous environmental surveys among larger feral fishes in water pollution studies. The current and future contribution of such species to studies in this area is substantial; smaller fish species were considered initially merely because of the size advantage in working with such animals in the testing laboratory.

In addition to the formal papers presented at the Symposium, I sought to provide as much opportunity as possible for a more informal exchange of ideas and recommendations addressing problems and prospects in the field. Thus the program included a panel discussion with each participant addressing a specific problem in the use of fish; a debate on the research merits of "Mouse versus Minnow" (suitably informal as it followed a congenial and pleasant dining interlude); and, on the 4th and final day, a consensus committee meeting in which 12 participants met to review the proceedings of the workshop and to discuss recommendations on present technical problems and future directions of research in the field. Regrettably, only a few of the question and answer periods following the presented papers were recorded, and these have been included because a great deal of valuable information was exchanged during these discussions.

It is most fitting that these Proceedings begin with a note of appreciation for Dr. Mearl F. Stanton, who initiated the use of a small fish species, the "zebra danny," in carcinogenesis studies when he published his experimental findings in the *Journal of the National Cancer Institute* in 1965. It is also fitting that this appreciative note be given by Dr. Clyde J. Dawe, formerly of the Section of Comparative Oncology, Laboratory of Pathology, National Cancer Institute, who served for many years as the project officer of the Institute-funded Registry of Tumors in Lower Animals located at the Smithsonian Institution in Washington, D.C., and whose considerable interest and expertise in comparative pathology extends from humans to marine invertebrates, a happy circumstance because he has recently retired to Woods Hole, Massachusetts.

I would like to extend my personal appreciation to Dr. Herman Kraybill, Scientific Coordinator for Environmental Cancer at the Institute, whose support of this Symposium was invaluable and to the Division of Cancer Cause and Prevention, which subsequently funded this project. My sincere thanks go also to Anne Marie Bozak, Division of Cancer Cause and Prevention, whose administrative talents were invaluable in the organization of the Symposium.

Karen L. Hoover, Sc.D.
Chairman

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[1] Some final revised papers of this Symposium were received October 4, 1983.

[2] Although the authors were unable to attend the Symposium, two invited papers by Dr. V. V. Khudoley of the USSR and Drs. K. Simon and K. Lapis of Hungary, respectively, have been included in these Proceedings.

Mearl F. Stanton, M.D.

1922–1980

Dedication: An Appreciation of Dr. Mearl F. Stanton[1]

Clyde J. Dawe[2]

Many if not most of you here today did not have the opportunity to know Mearl Stanton, to whose memory this meeting is dedicated. So I will try to give you a brief word picture of him, without repeating too much of what has been said in the November 1981 issue of the *Journal of the National Cancer Institute* (*1*). Mearl served as Editor in Chief of the *Journal* from 1967 to 1974, and the issue cited above was dedicated to him in recognition of his laboratory contributions to cancer research as well as his editorial skills. The cover carried a photograph of Mearl over a backdrop of amosite asbestos fibers symbolic of his most absorbing interest, fiber carcinogenesis, during the last decade of his life. I recommend to all of you a reading of Dr. Harold L. Stewart's colorful perceptions of Mearl's career as recounted in that issue

Mearl's interests were wide-ranging, and the reason we are thinking of him today is that he was the one who demonstrated that, if you are going to use fish instead of mice or rats, you do not have to use big, beautiful, and expensive fish like the rainbow trout to prove the hepatocarcinogenicity of a chemical (*2*). He found that you can do much the same thing with a small, inexpensive, but also beautiful fish such as the "zebra danny," *Brachydanio rerio*. It was a matter, I suppose, of thinking small in a big way; a big way because, in the years that have ensued, perhaps a score of other investigators have confirmed and extended Mearl's work by using about 15 species of small aquarium fishes and an even larger number of chemicals. Mearl himself went on to show that cycasin, too, is a hepatocarcinogen for zebra dannies (*3*), but he stopped there. I think he felt that he had pointed the way and others could be depended on to take it further. The existence of this workshop indicates that he was right.

Mearl did not derive his interest in fish as experimental subjects from any interest in angling. He told me that he had never done any fishing, and his attitude toward fishing seemed to be about the same as that you might expect a fish to have. He did not "take to the notion"; but if he had, he would have been a great fisherman. Like the idealized Compleat Angler of Walton's book, Mearl was a contemplative person who liked to think out the approach to his objective before plunging in over his boot tops. In 1963, he was persuaded with some effort to accompany Frank Schwartz and me on a survey of the fish population in Hoop Pole Cove in Deep Creek Lake, Maryland. The fish were killed by rotenone poisoning, a method still practiced in some places. We were soon knee-deep in dead fish, and, in those we had time to necropsy before *odor mortis* set in, we found several cholangial neoplasms in white suckers and multiple hepatocellular neoplasms in 1 brown bullhead (*4*). On the way home, we discussed the possible etiology of these neoplasms, listing motor boat exhausts and the pesticides and herbicides then in wide use in the Deep Creek Lake area. We even wondered whether rotenone could be carcinogenic because it had been used repeatedly to sample the fish population in this lake. We looked it up the next day and found that, structurally, it was similar to 3-methylcholanthrene. It was not until 10 years later that Gosalvez and Merchan (*5*) showed that it readily induced mammary tumors in rats. To this day we do not know whether serious dangers are involved in taking fish samples from waters in which rotenone is used, or whether rotenone had anything to do with the liver tumors we saw. We do know that the use of rotenone as an insecticide has been largely abandoned.

Anyway, to continue the story, Mearl and I talked off and on for several weeks about liver neoplasms in rainbow trout, white suckers, and brown bullheads. Then one day several small glass aquaria containing zebra dannies appeared in Mearl's laboratory. At first he put sand mixed with concentrated outboard motor exhausts in the tanks, but it proved too toxic for the fish, and they all died. Then, perhaps because diethylnitrosamine was coming into use with rodents, he tried that, and you know the rest of the story. Less than 6 months later he was showing me sections of hepatocellular neoplasms in dannies. Later, Mearl (*2*) insisted that the massacre at Deep Creek Lake had nothing to do with the rationale of his experiment (*6*). It was a simple case of economic necessity and esthetic considerations. He did not like to see beautiful trout "wasted." In any case, the facts testify to his thoughtful and conservative approach to a problem. After all, he could have done the obvious thing and started a brown bullhead hatchery costing millions.

Those of you who are familiar with and have benefitted from the services of the Registry of Tumors in Lower Animals at the Smithsonian will be interested to know that Mearl Stanton played an important part in the establishment and support of that Registry. He served as assistant or co-project officer from the beginning of the Registry in 1965 until just a year or so before his death. He chose to stay in the background pretty much, unless help was needed either with a diagnosis or in the form of support for continuation of the contract or for new activities. Then he offered a willing hand. One of the first experimental projects undertaken by John Harshbarger after he joined the Registry was a collaborative attempt with Mearl and George Cantwell to induce tumors in crayfish, using dimethylnitrosamine (*7*). That time it did not work, and it remains a mystery why crustaceans appear disinclined to develop neoplasms. There is still no example of a crustacean neoplasm in the Registry's files.

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[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Section of Comparative Oncology, Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Public Health Service, Department of Health and Human Services, Bethesda, Maryland 20205. *Present address:* Clyde J. Dawe, M.D., Ph.D., 59 School Street, Woods Hole, Massachusetts 02543.

Mearl was large of body as well as mind. Treading softly in his interactions with people, he was nonetheless difficult to push if he did not want to be pushed. His humor was often expressed in tongue-in-cheek statements designed to test people's reactions. Inclined to be pensive, informal, and relaxed, he also displayed great patience and courage in the face of his illness, the Shy-Drager syndrome, a rare degenerative neurological disease affecting the autonomic and motor systems. Ironically, recent studies by Dr. J. H. Robbins, starting from the cancers in patients with xeroderma pigmentosa, have led to speculation that a defect in DNA repair mechanisms may be a basis for the Shy-Drager syndrome, as in the central nervous system degeneration in patients with xeroderma pigmentosa. One never knows where a piece of research will lead. I suspect Mearl would have grinned sardonically at the twist of events in xeroderma pigmentosa research, and I know he would be amused and delighted to see where his little experiment with dannies and diethylnitrosamine has led.

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SESSION I

Proposed Species for Carcinogenicity Testing In Vivo

Chairman: **Clyde J. Dawe, M.D., Ph.D.**

Characteristics of a Fish Colony of *Poeciliopsis* and Its Use in Carcinogenicity Studies With 7,12-Dimethylbenz[*a*]anthracene and Diethylnitrosamine[1,2]

R. Jack Schultz *and* Mary E. Schultz[3,4]

ABSTRACT—The viviparous fish *Poeciliopsis* from northwestern Mexico is characterized according to its potential in cancer research. A colony of these fishes started in 1961 includes inbred strains of *P. lucida* (M61-9, M61-31, M61-35, and S68-4), *P. monacha* (S68-4 and S68-5), and *P. viriosa* (M65-23), which have been demonstrated by electrophoresis and tissue graft analysis to be homozygous. All-female species of hybrid origin are represented by 12 clones which, although highly heterozygous, are genetically identical or isogenic and readily accept within-clone tissue transplants. Two studies are cited in which hepatic tumors were chemically induced. In one, liver neoplasms were induced in *P. lucida* and *P. monacha* by repeated short-term exposures to an aqueous suspension of 5 ppm 7,12-dimethylbenz[*a*]anthracene. In the other, dose responses to the carcinogen diethylnitrosamine (DENA) were compared among 4 inbred strains, 3 hybrids from crosses between inbred strains, and 2 wild stocks of *P. lucida*. The incidence of hepatic tumors gradually increased from 0 to 93% depending on the concentration of the compound and the number of exposures, but no significant difference was found among the inbred strains, their hybrids, or the wild stocks. Hepatic tumors, initially induced with DENA have been successfully transplanted into the muscle tissue and abdominal cavity of members of the same strain. — Natl Cancer Inst Monogr 65: 5-13, 1984.

The release of pollutants into our natural waters from time to time produce dramatic kills of aquatic life leading to public outrage, subsequent fines, and additional legislation. In this study, we address a more subtle but related issue, one that concerns sublethal levels of pollutants with long-term effects which may, nonetheless, be equally devastating.

Various substances which have entered our natural waters at seemingly sublethal levels produce cancer as well as a host of other diseases having the capacity to kill eventually, debilitate, or sterilize the organisms exposed to them. Furthermore, commercial species can be made unpalatable by unsightly lesions. For example, in heavily polluted zones, such as certain areas of the Great Lakes of North America, 30–50% of the fish have tumors (*1*). The total impact of cancer on fish populations, however, is not easily assessed because most fish live and die without being seen by man. Fish that die before capture or small fish, either young or stunted, that pass through the net may add an uncalculable cost.

Quantitative evaluation of the carcinogenic properties of aquatic pollutants have been hampered by a lack of appropriate experimental animals from aquatic habitats for which the genetic and biological properties have been explored.

In this paper, we considered criteria that may be important in selecting organisms for testing aquatic pollutants; we have also examined the viviparous fish *Poeciliopsis* as a potential test animal in 2 studies in which the carcinogens DMBA and DENA were administered.

HISTORY AND CHARACTER OF THE UNIVERSITY OF CONNECTICUT FISH COLONY

The genus *Poeciliopsis* comprises guppy-sized, live-bearing fishes found in the fresh and brackish waters of northwestern Mexico. Our original interest in the group developed from the discovery that several of the species give birth to only female offspring (*2*). Over the years, investigators (*3*) at this laboratory have been engaged in a series of studies with these fishes involving their reproductive biology, genetics, population dynamics, ecology, and behavior.

Currently 11 species of *Poeciliopsis* are maintained, 5 of which are bisexual (i.e., they produce offspring of both sexes) and 6 that are unisexual and produce only female offspring. Among the bisexual species, 3 inbred strains of *P. lucida* were started in 1961 and are now in their 40th to 50th generation; 1 inbred strain of *P. viriosa,* started in 1965, is 29 generations old; 1 of *P. lucida* and 2 of *P. monacha,* started in 1968, are now 20 inbred generations old. More recent strains, as well as wild stocks, that are no more than 1 or 2 generations old are also available. The inbred strains, maintained mostly by brother–sister matings, are homozygous to the extent that all 24 electrophoretic loci examined are homozygous (*4*), and tissue grafts of heart, spleen, liver, or dermal tissues into the musculature of any member of the same strain are readily accepted (*5*).

The unisexual species consist of multiple clones. Up to 22 have been identified in nature from 1 species and 18 from another (*5, 6*). A total of 12 of these are maintained in the laboratory. Tissue grafts are readily accepted among

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ABBREVIATIONS: DMBA = 7,12-dimethylbenz[*a*]anthracene; DENA = diethylnitrosamine; H & E = hematoxylin and eosin.

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[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Supported in part by grant R805195 from the United States Environmental Protection Agency.

[3] Biological Sciences Group, University of Connecticut, Storrs, Connecticut 06268.

[4] We thank Mrs. Ellie DeCarli for her assistance with typing the manuscript.

members of the same clone, testifying to the fact that, although these unisexuals are of hybrid origin (*7–9*) and are highly heterozygous (*4, 10*), members of the same clone are genetically identical or isogenic to each other. Between-clone grafts are rejected within 7 to 10 days.

The biological properties of these fish offer some specific advantages as experimental animals: 1) Specific pairs of known lineage can be maintained in 2-gallon tanks and their young reared as intact broods, requiring only 1 gallon of water/adult fish. 2) They can endure a wide range of temperatures from near freezing to 40° C and can, in fact, accept a fairly rapid change within this range (*11*). 3) Young are born at 8- to 12-day intervals, at a size of 7–8 mm, usually in lots of 8–20, though sometimes more than 30 are produced. 4) Adults mature at 2½ to 3 months and have a life-span of about 2 years. 5) Pregnant females carry active sperm for up to 6 months and can continue reproducing without being remated. 6) Our laboratory stocks are free of parasites that might otherwise confuse a histological picture or lead to premature death of test animals. 7) The origin of the stocks is from the base of the Sierra Madres, from headwater streams free of industrial pollution and any possible selection for resistance to pollutants. 8) Recently collected strains do not have to undergo several generations of selection before they can live in the laboratory. 9) Our inbred strains have not become genetically imbalanced as a result of selection for specific traits as has occurred among hatchery fishes and domesticated rats and mice. Unintentional selection for survival in the laboratory and even inbreeding to a point of complete homozygosity does not appear to have influenced their capacity to survive in nature. Each spring we stock one of our local Connecticut lakes with *Poeciliopsis* where they grow and reproduce in great numbers until winter. 10) Most of the tumors we have induced so far (nearly 200) have been liver tumors, yet no liver tumors have appeared in any of the 400 controls we have examined, which indicates that our base level is either extremely low or zero. 11) The fish are small enough that they can be treated in small clean glass containers wherein specific quantities of the carcinogens can be delivered.

The unisexual forms themselves offer a wealth of experimental opportunities because of their hybrid origins and reproductive mechanisms. Three of them are triploids and reproduce by gynogenesis; 3N (triploid) eggs are produced which are then stimulated into development by sperm provided by other species. No gametic fusion takes place, and, therefore, none of the paternal traits occur in the progeny. Tissues can be grafted among the daughters or back to the mother; grafts to or from the father are rejected. The other 3 unisexual forms are diploid and reproduce by hybridogenesis. In these, only the maternal genome is transmitted to haploid eggs (*3, 7, 8, 12*). Fertilization in nature by males of 3 species perpetuates 3 diploid hybrids all with the same maternal genome (*P. monacha*) but different paternal genomes (*P. lucida, P. occidentalis*, or *P. latidens*). Inasmuch as only the maternal genome is inherited clonally and the paternal genome is replaced for each generation, we refer to these as hemiclones. In the laboratory, wild paternal genomes for subsequent generations can be replaced with genomes from other species or from inbred lines, which permits tissue grafts to be made among individuals of the same hemiclone who have the same father. Hybridogenetic unisexuals can also be synthesized in the laboratory by crossing *P. monacha* with a male of 1 of 3 other species (*9*). Thus both mechanisms allow us to produce unlimited numbers of individuals that are heterozygous but genetically identical to each other. The long-range potential of such animals for investigators studying the inducibility of cancer and its rate of development in different genetic backgrounds is extensive. To select only one of many possible combinations, a researcher can graft a tumor induced in an inbred strain of *P. lucida* into a *P. monacha-lucida* unisexual whose paternal genome derives from this same strain. For a control, the same tissue can be grafted into a homozygous sister of that donor. Recently, we successfully transplanted small pieces, less than 1 mm^3, of a DENA-induced liver tumor into the dorsal musculature of 3 isogenic recipients. The growth of the tumor tissue was prolific, and all 3 fish were moribund after 3 months. Tumor tissue from 1 of the initial recipients was transplanted into the abdominal cavity or dorsal musculature of 8 additional isogenic fish; after 1 month, conspicuous growth can be observed. These studies are in progress in our laboratory.

TEST PROCEDURES

Development of a system for administration of carcinogens that allows delivery of specific quantities is essential in the determination of effective dose levels, length of exposure periods, and number of exposures. When carcinogens are placed in the food, intake can vary depending not only on how much food the fish eat but also on how much food falls to the bottom and dissolves in the water, later to be absorbed through the gills in unknown quantities. Chemicals dissolved directly in the aquarium water may be taken up by materials in the tank; they may either accumulate with repetitive treatments or break down into other compounds. Injection of carcinogens directly into the fish is difficult for one to quantify in small specimens because often they do not have enough tissue to absorb a dose of sufficient size to be measured accurately. This is especially true of young fish. Embedded pellets made of treated paper coated with paraffin also encounter a fish-size problem and, in addition to this, pose some question about the rate of uptake by the recipient. Adding the carcinogen to a continuous flow water system removes several of the above disadvantages, but the method is not suitable for use with expensive compounds. Furthermore, it requires the disposal of large volumes of polluted water. Any of the above treatment methods pose health hazards for the aquarists when highly carcinogenic materials are used.

In our studies, the fish are placed in clean beakers containing 400 cm^3 of dechlorinated tap water to which the carcinogen has been added. This is all assembled under a hood where the fish then remain in the dark and without food for a specific period ranging between 5 and 24 hours. After treatment, the fish are returned to their original aquaria where they receive normal care until the next

TABLE 1.—*Induction of hepatic tumors in* P. lucida *and* P. monacha *exposed to 5 ppm DMBA for 3–4 periods varying in length from 5–20 hr*[a]

Species	Strain	Age at initial dose, mo	No. of fish treated	Latency period, mo[b]	Exposure regimen, hr[c]	No. of fish surviving	Tumor incidence
P. lucida	M61-31	1	18	8	20, 20, 20, 20	3	2/3
		2	3	7	20, 10, 5	2	1/2
	M61-9	1	7	7	20, 10, 5	1	0/1
		5	3	8	20, 10, 5	2	2/2
		7	6	7	20, 20, 20	5	1/5
	VL78-18	2½	8	8	20, 10, 5, 5	3	3/3
	S68-4	7	11	8	20, 20, 20	9	6/9
P. monacha	S68-4	1	38	7	20, 10, 5, 5	21	7/21

[a] *See* (*15*).

[b] Period is defined as that allowed for tumor development beginning at time of initial exposure to carcinogen.

[c] Repeated exposures of varied length are listed in sequence and were given at 1-week intervals.

treatment a week later. With this simple method, any number of exposures can be administered at any desired concentration. Our results demonstrate that continuous exposure is not required for tumor induction and that the number of exposures and the concentration of the carcinogen influence the frequency of tumors among the treated fish.

INDUCTION OF HEPATIC TUMORS WITH 7,12-DIMETHYLBENZ[*a*]ANTHRACENE

The polycyclic aromatic hydrocarbon DMBA has been amply demonstrated to be carcinogenic to mice, rats, hamsters, and guinea pigs (*13*). Initial attempts by Pliss and Khudoley (*14*) to induce tumors in fish with DMBA, however, were unsuccessful. In previous studies, the compound was administered to guppies, *Poecilia reticulata,* by: 1) injections, 2) implantations of paraffin-lanolin-DMBA pellets, 3) skin applications, and 4) feeding, none of which resulted in tumors. We (*15*) recently induced liver neoplasms in *P. lucida* and *P. monacha* by repeated short-term exposures to an aqueous suspension of DMBA. In 1 series of experiments, the fish were exposed to 5 ppm DMBA during 3 to 4 successive periods of 20 hours or for 1 period of 20 hours followed by shorter periods of 10 and 5 hours (table 1). Tumors were found in the liver of 22 of the 46 fish that survived the 7–8 months allowed for tumor development; most were primary liver tumors (figs. 1–4); 2 were lymphosarcomas involving the kidneys, spleen, pancreatic tissues, heart, and liver.

A lower concentration of DMBA, 0.25 ppm, did not induce tumors in any of the 230 survivors of 295 fish receiving 2 to 4 exposures (20 hr), nor did any of the 120 controls we monitored in the DMBA studies develop tumors.

Fish younger than 2 weeks old could not survive the toxic effects of DMBA at concentrations of 0.5–5.0 ppm; on the other hand, 0.25 ppm did not induce tumors. By lowering the treatment time to only 6 hours, the toxic effects of 5.0 ppm DMBA were sufficiently reduced to allow 5 successive treatments. Even with this short exposure time, hepatic tumors developed in 25 of the 60 fish surviving the 6- to 7-month period allowed for neoplastic development (table 2).

TABLE 2.—*Induction of tumors*[a] *in* P. lucida *and* P. monacha *exposed to 5 ppm DMBA for 6-hr periods at 5 weekly intervals*[b]

Species	Strain	Age at initial dose, wk	No. of fish treated	No. of fish surviving	Tumor incidence
P. lucida	M61-31	4	28	27	8/27
	LM78-13.8	1	15	13	9/13
	VL78-18	1	18	12	4/12
P. monacha	S68-4	6	15	8	4/8

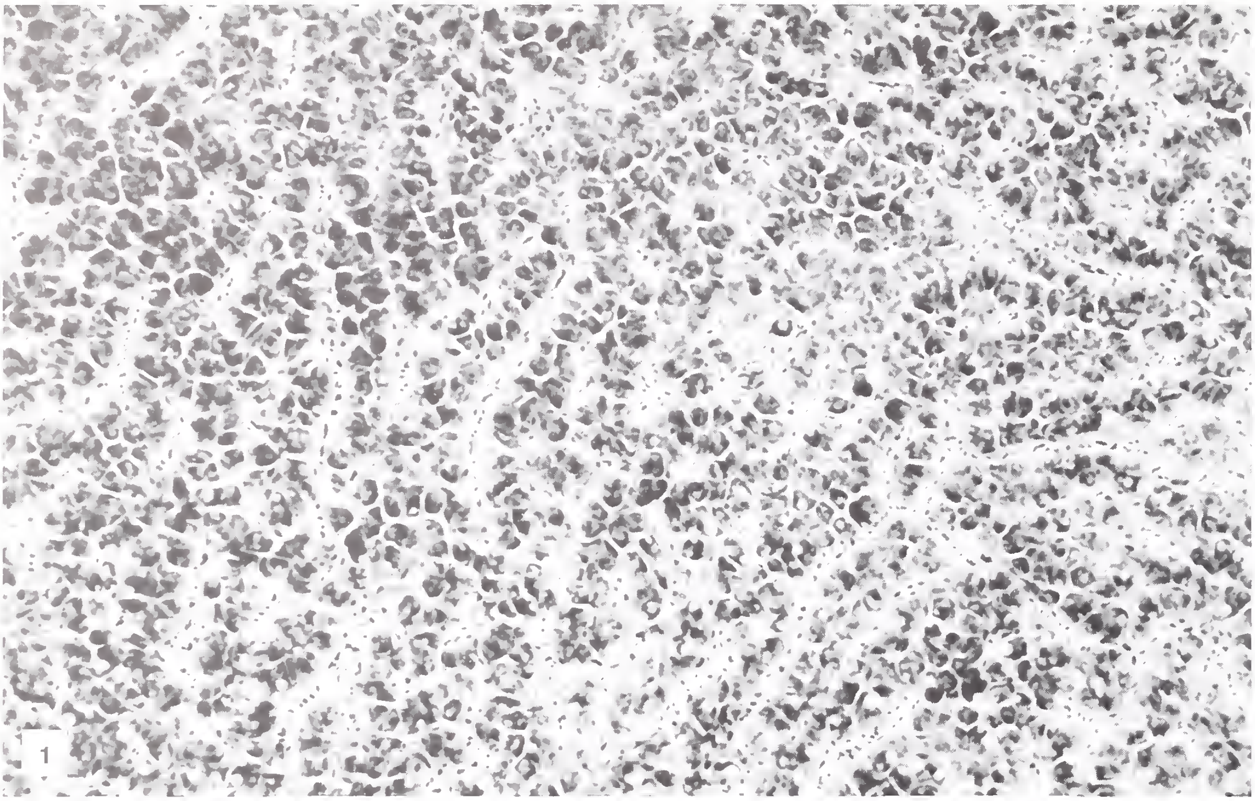
[a] Tumors include 23 hepatocellular carcinomas and 2 lymphosarcomas. *See* (*15*).

[b] An 8-month period was allowed for neoplastic development beginning at the time of initial exposure to the carcinogen.

WILD VERSUS INBRED STRAINS

Inbred strains, especially mice, rats, and hamsters have been popular for testing carcinogens and for toxicological studies; they offer advantages which are not immediately available in most other vertebrates: 1) They circumvent the nuisance of collecting and the pitfalls of identifying wild species. 2) Rearing techniques have been developed and lines have been bred to produce sufficiently well in captivity to insure delivery in quantity. 3) Greater repeatability of results is anticipated from inbred stocks than from poorly defined and uncontrolled gene pools.

However, results from bioassays in which inbred animals are tested are occasionally challenged with claims that especially sensitive strains were used. Certainly, between-strain differences in cancer susceptibility exist (*16, 17*). Investigators often use crosses between inbred strains to take advantage of the increased heterozygosity in an F_1 generation; when a range of genotypes is wanted, F_2 hybrids are recommended (*18*). Interstrain crosses, however, may not provide genotypes comparable to wild types. The process of domestication, either by chance or by selection, may exclude the same wild-type genes from both



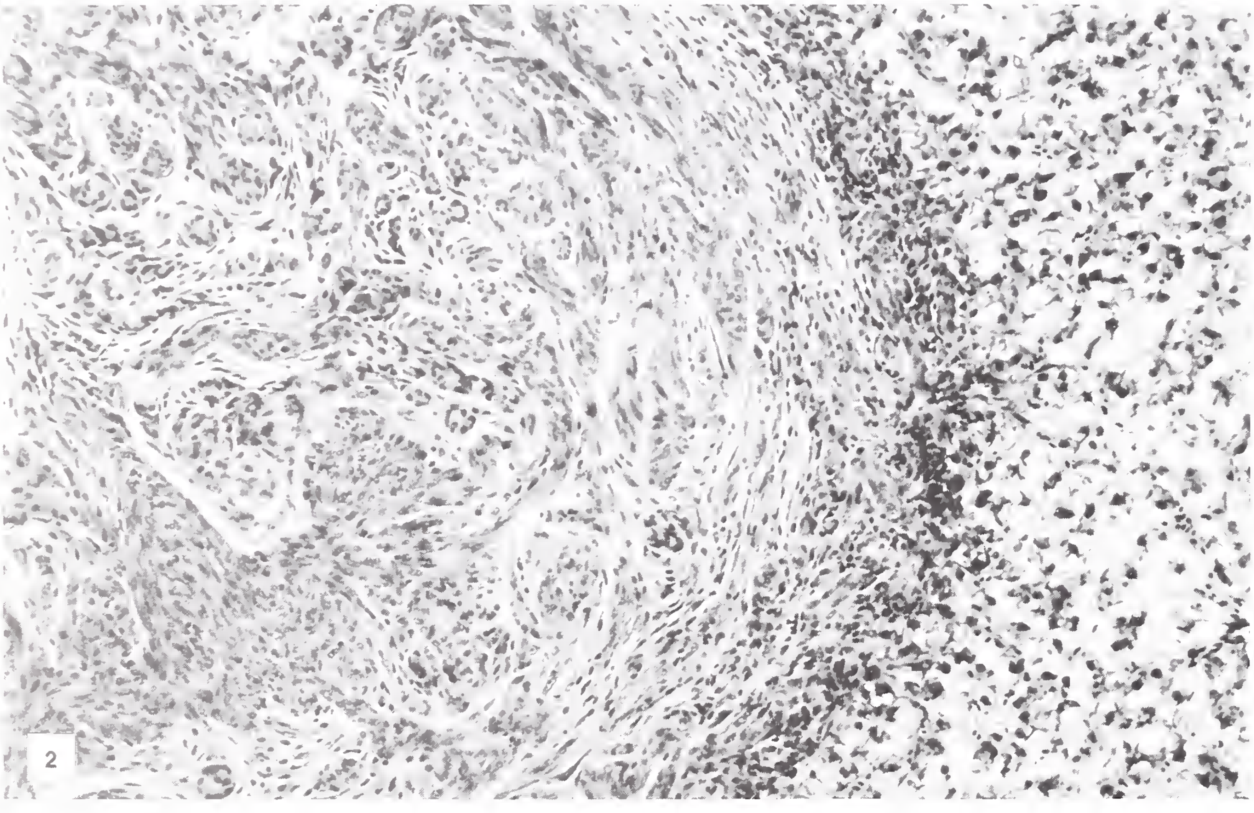
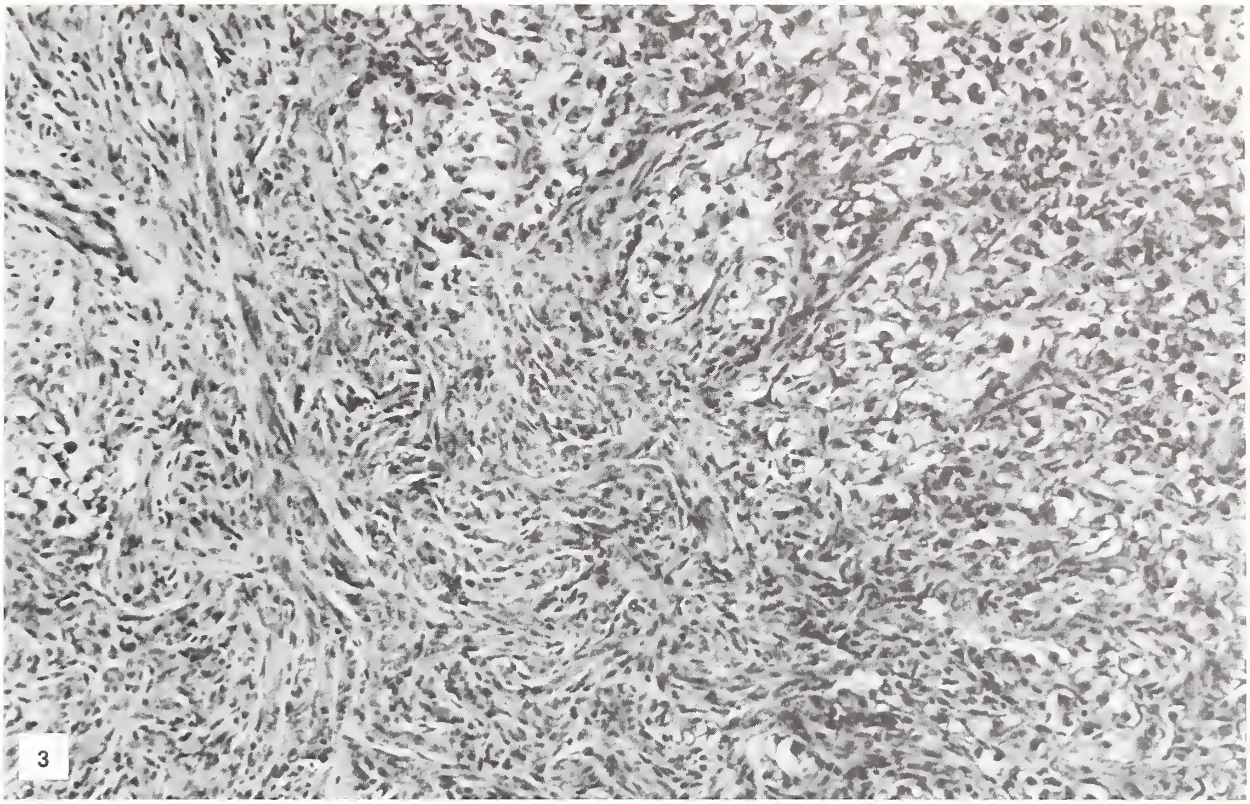


FIGURE 1.—Liver of a 10-mo-old untreated fish. Hepatic parenchyma is separated by vascular sinusoids into cords, two-cell layers in thickness, interspersed with rosettes composed of 5–7 cells. H & E. × 300

FIGURE 2.—Nodular-type hepatocellular carcinoma composed of poorly differentiated basophilic cells with scattered tubule formations. Normal hepatic parenchyma is adjacent (*right*). Five ppm DMBA for 5 exposures for 6 hr. H & E. × 300



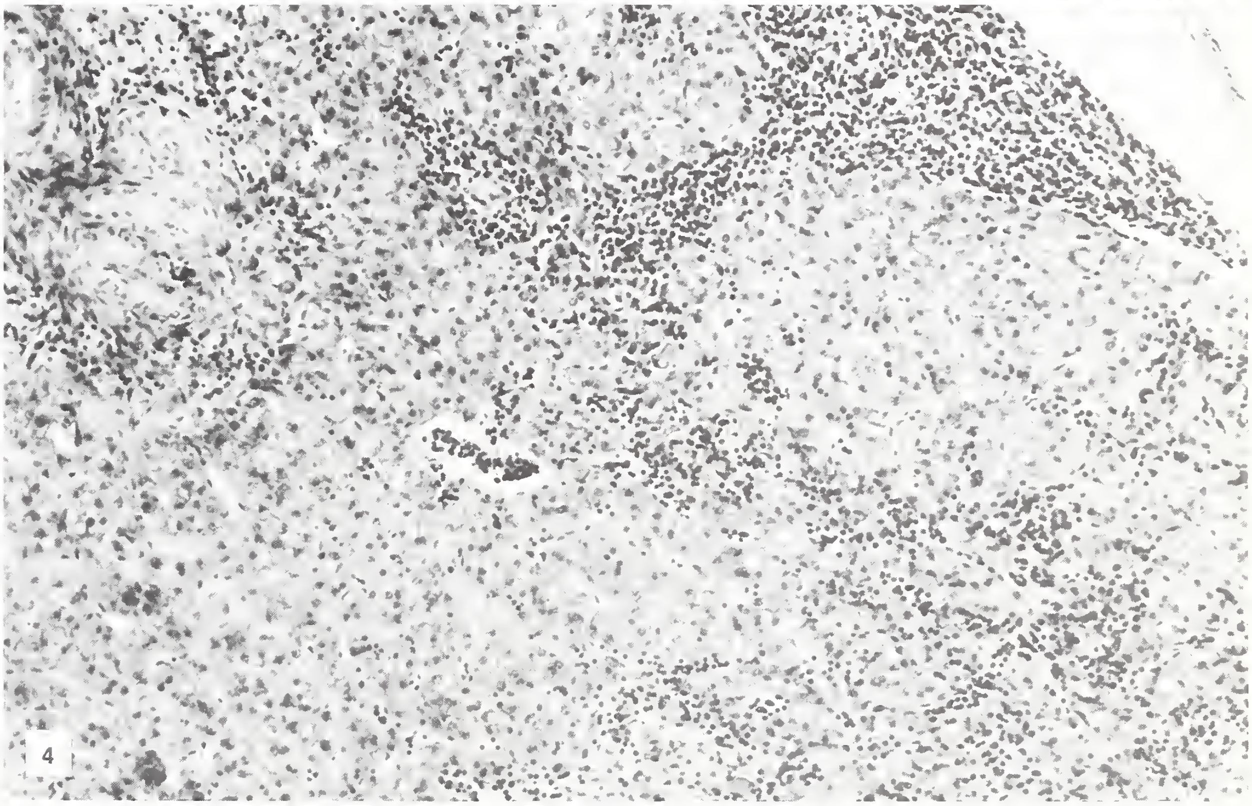


FIGURE 3.—Hepatocellular carcinoma with irregular boundaries extending into the normal parenchyma was composed of poorly differentiated basophilic cells. Five ppm DMBA for 4 exposures for 20 hr. H & E. × 300

FIGURE 4.—Liver lymphosarcoma showing infiltration of dense staining round or oval neoplastic cells and destruction of hepatic parenchyma. Neoplastic cells occurred in greatest abundance in the kidneys and spleen of this fish. Five ppm DMBA for 4 exposures for 5, 5, 10, and 20 hr. H & E. × 300

strains; by a combination of two laboratory artifacts, a third is produced, none of which could survive in nature (*19*).

To examine the suitability of *Poeciliopsis* as a test animal for aquatic pollution, we (*19*) compared response to DENA in *P. lucida* among 1) strains that were inbred for up to 43 generations; 2) crosses between inbred strains; and 3) wild stocks, 1 generation removed from nature.

Liver carcinomas have been induced in other aquarium fishes, e.g., the guppy, *P. reticulata* (*14*); zebra danio, *Brachydanio rerio* (*20*); and the medaka, *Oryzias latipes* (*21*); by the addition of DENA directly to the aquarium water and continuous exposure for 8 weeks.

To test for differential responses to the carcinogen among the various strains of *Poeciliopsis*, we needed a quantitative system of delivery. In this study (*19*), DENA was administered to the fish in clean beakers of water at concentrations of 25, 50, and 125 ppm for 3 to 5 periods of 23–24 hours, separated by 1-week intervals. After each exposure, the fish were returned to their original aquaria.

Primary hepatocellular carcinomas were induced in fish exposed to 125 ppm DENA three to five times (figs. 5, 6). The incidence increased from 58 to 93% with the number of exposures. Tumors were not found in fish that received the lowest dose of 25 ppm three times, but a low incidence or 3–10% occurred in fish exposed three to five times at 50 ppm (table 3).

Having demonstrated that concentrations of DENA at 25, 50, and 125 ppm, when administered in 3, 4, or 5 exposures provided a range of tumor inducibility from 0 to 100% (table 3), we then used the system to test 4 inbred strains of *P. lucida*, 3 intercrosses between them, and 2 wild stocks. No significant difference of tumor incidence occurred among the various genotypes listed in table 4 ($P<0.005$, chi-square tests of homogeneity). No tumors were found in fish exposed to the lowest dose, i.e., for three times at 25 ppm, but at the highest dose, 125 ppm five times, primary liver tumors ranged between 84 and 100% in all strains. We found tumors in 6 of 198 fish exposed to the carcinogen at 50 ppm three times. Two of these were in progeny of interstrain crosses, 1 was in an inbred strain, and 3 were in the wild stocks.

Because exposure at 125 ppm five times is almost totally effective and three times at 25 ppm is ineffective, treatment for three times at 50 ppm, which has a 3% tumor incidence, marks the lower edge of effective treatment levels. If between-strain differences in resistance to the carcinogen existed, this level of treatment should have revealed them. However, as a supplement to this series, we exposed nearly 250 additional fish to 1 or 2 more doses of DENA at 50 ppm (four and five times) and 1 or 2 fewer doses at 125 ppm (three and four times). Two inbred strains, 1 interstrain cross, and 2 wild stocks were used. Additional exposures (more than three times) at 50 ppm increased the incidence of tumors, and fewer exposures (less than five times) at 125 ppm decreased the incidence, but neither treatment revealed any differences in susceptibility among strains.

The absence of response differences to DENA among wild stocks, inbred strains, and interstrain crosses of *P. lucida* is in contrast to observations in other animals. Interstrain differences in tumor-inducibility in mice are well documented (*16, 17, 22, 23*).

Various strains of *Poeciliopsis* may not show differences in response to carcinogens because their laboratory culture has not been accompanied by any form of directed selection (*19*). Domestication of other animals usually involves the following processes: 1) selection to thrive under man-made conditions, 2) selection of traits of interest to men, and 3) fixation of desirable traits by the organisms made homozygous. All of these procedures could effectively reduce the number of alternative developmental and physiological pathways. Although such organisms may prosper under uniform culture conditions, they may lack the diversity of response (genetic homeostasis) necessary to cope with wild environments or stress.

New stocks of *Poeciliopsis* adapt immediately to the laboratory and over the years no selection has been practiced. Response to temperature stress (*11*), reproductive vigor, and other measures of fitness (*24*) do not differ between inbred and newly acquired stocks. Inbreeding depression, characteristic of other animals, does not present a problem in the establishment of new lines. Why *Poeciliopsis* possesses these attributes may reside in its way

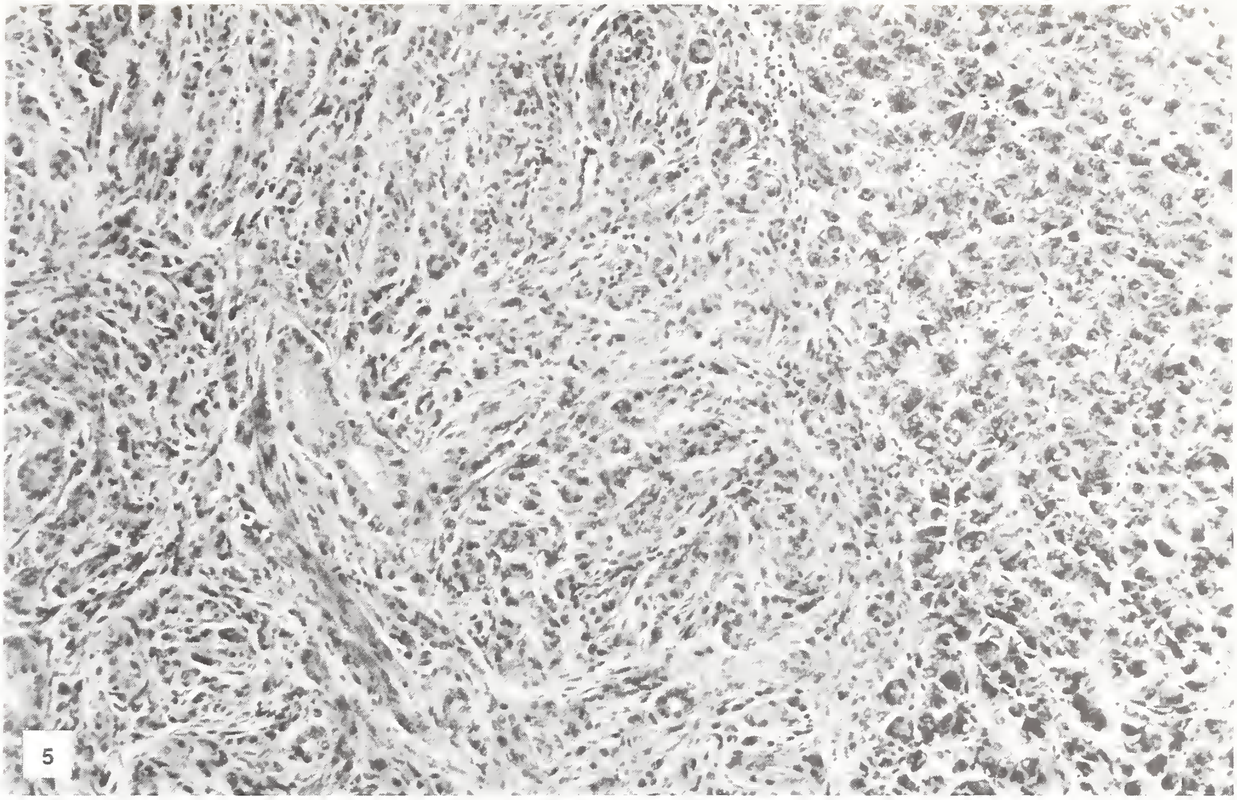
TABLE 3.—*Liver tumors in* P. lucida *at various dose levels and exposures of DENA*

Dose level, ppm	No. of exposures	No. of fish examined[a]	Incidence of neoplasms[b]			
			Eosinophilic nodules[c]	Primary carcinomas	Mortality	
					No.	Percent
25	3	131	0.06	0.00	36/180	20.0
50	3	192	0.16	0.03	46/255	18.0
50	4	50	0.20	0.10	11/61	18.0
50	5	52	0.20	0.04	14/73	19.2
125	3	31	0.14	0.58	13/48	27.0
125	4	40	0.10	0.80	14/62	22.6
125	5	89	—	0.93	99/192	51.6
Control	—	375	0.00	0.00	66/375	17.6

[a] Histological data on inbred lines, intercrosses, and new laboratory lines are combined. *See* (*19*).

[b] Incidence was calculated from No. of fish with tumors/No. of fish examined histologically.

[c] Eosinophilic nodules are composed of highly differentiated hepatocyte-like cells that apparently were nonmalignant.



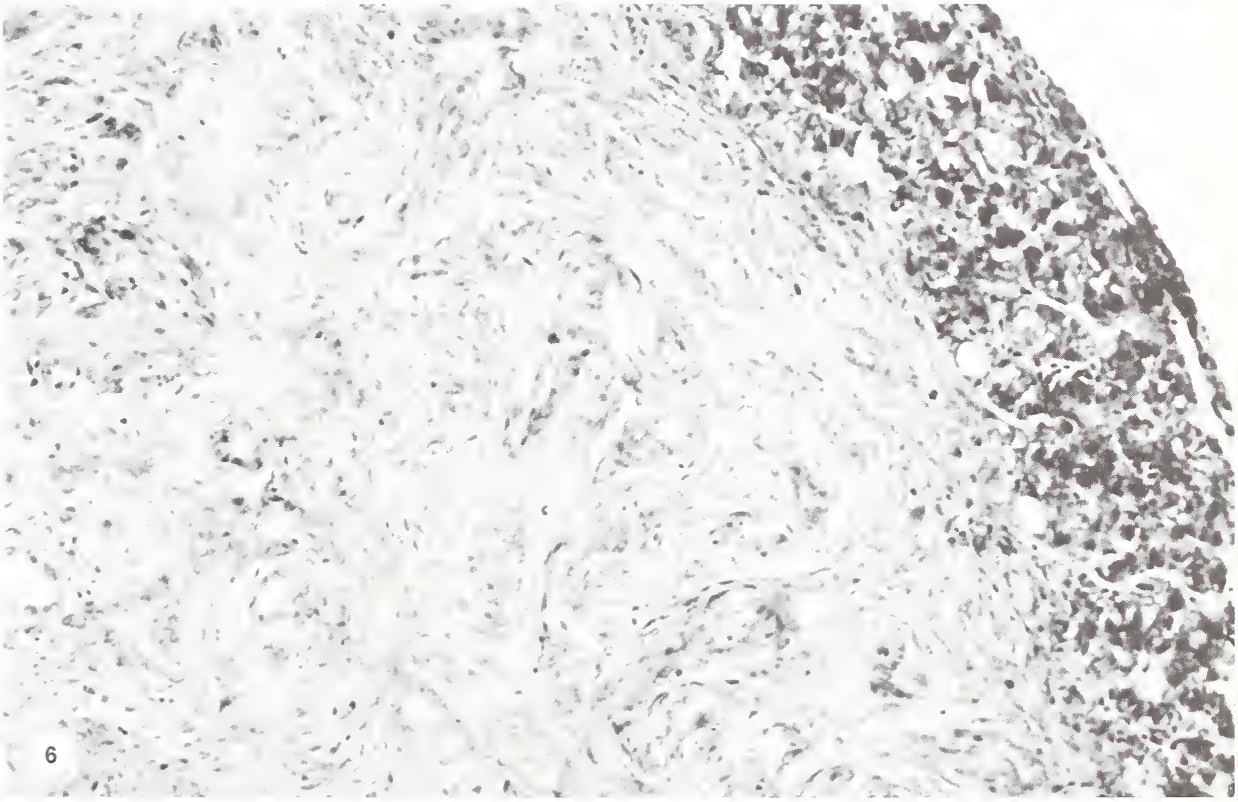


FIGURE 5.—Nodular-type hepatocellular carcinoma composed of poorly differentiated basophilic cells with conspicuous tubule formations. A 125-ppm DENA dose for 5 exposures for 24 hr. H & E. × 300

FIGURE 6.—Large carcinoma replacing all but an outer narrow margin of normal liver parenchyma after a 9-mo latent period. Neoplastic tissue is lightly basophilic and shows frequent division figures. A 125-ppm DENA dose for 5 exposures for 24 hr. H & E. × 300

TABLE 4.—*Liver tumor induction by DENA in inbred lines, intercrosses, and wild stocks of P. lucida*[a]

Lines	Strain	Dose level, ppm	No. of exposures[b]	Tumor incidence[c]	Mortality
Inbred	M61-31 PC (F_{41})	25	3	0/31	2/33
		50	3	0/21	6/33
		125	5	16/19	18/37
		Control	—	0/51	4/51
	M61-35 PC (F_{39})	25	3	0/15	2/18
		50	3	1/19	5/28
		125	5	7/7	8/15
		Control	—	0/30	7/30
	M61-9 PC (F_{43})	25	3	0/21	1/23
		50	3	0/29	1/35
		125	5	11/12	5/17
		Control	—	0/30	3/30
	S68-4 PC (F_{20})	25	3	0/28	3/35
		50	3	0/16	3/18
		125	5	10/11	12/24
		Control	—	0/63	7/63
Intercrosses	M61-31 PC × M61-35 PC	25	3	0/9	1/14
		50	3	0/4	3/8
		125	5	6/6	14/21
		Control	—	0/22	4/22
	M61-31 PC × S68-4 PC	25	3	0/13	2/16
		50	3	1/32	5/39
		125	5	7/8	5/14
		Control	—	0/50	8/50
	S68-4 PC × M61-31 PC	25	3	0/6	5/13
		50	3	1/12	4/16
		125	5	5/5	11/16
		Control	—	0/40	7/40
Wild stocks	VL78-18 PC (F_1)	25	3	0.3	15/18
		50	3	1/29	6/36
		125	5	11/11	10/22
		Control	—	0/50	15/50
	LM78-13.8 PC (F_1)	25	3	0/5	5/10
		50	3	2/30	11/42
		125	5	10/10	16/26
		Control	—	0/39	11/39

[a] *See* (*19*). Controls were not exposed to DENA.

[b] Each exposure was for 23–24 hr at 1-wk intervals.

[c] Incidence was calculated from No. of fish with tumors/No. of fish examined histologically; well-developed hepatocellular carcinomas, eosinophilic nodules were excluded.

of life and evolutionary history. This fish species lives in desert streams subject to considerable fluctuations in water level that result in fluctuations in population size and loss of genetic variation. A certain amount of inbreeding may have been part of its way of life for many years, such that some degree of homozygosity has already developed. The process may have been gradual enough to avert the genetic imbalance and loss of homeostasis generally associated with intensive laboratory selection and inbreeding.

If the response of *Poeciliopsis* to DENA and DMBA in these 2 studies is characteristic of the responses one might expect in tests with other carcinogens and toxicants, these fish could provide an outstanding model for the study of aquatic pollutants.

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Usefulness of the Hermaphroditic Marine Fish, *Rivulus marmoratus*, in Carcinogenicity Testing[1, 2, 3]

Christopher C. Koenig[4] *and* Michael P. Chasar[5, 6, 7]

ABSTRACT—Hepatocellular carcinoma and other pathological changes were induced in the self-fertilizing hermaphroditic fish *Rivulus marmoratus* by aqueous exposure to 15, 30, and 45 ppm diethylnitrosamine (DENA). Fish exposed as larvae for 12 weeks and adults exposed for 5 weeks showed rates of hepatocellular carcinoma as high as 76.5 and 31.3%, respectively, when examined 1 year later. Fish exposed as embryos in various stages of development for 1 week to 10, 32, 100, 320, and 1,000 ppm DENA were without neoplasms when examined 1 year later. Other pathological changes of rivulus livers were cholangioma, adenofibrosis, and granuloma with incidences as high as 38.9, 88.9, and 61.1%, respectively. Simple rearing methods were developed and relevant biological data pertaining to reproduction, embryonic development, and subsequent growth of *R. marmoratus* are discussed. Advantages and disadvantages of the use of *R. marmoratus* as a carcinogenicity test animal are assessed. — Natl Cancer Inst Monogr 65: 15–33, 1984.

Much early work on neoplasms in fish grew out of an unexpected outbreak of liver cancer in cultured rainbow trout, *Salmo gairdneri,* from 1957 to 1960 (*1*). The cause of the disease was traced to an aflatoxin-contaminated diet. Subsequent studies demonstrated not only the potent carcinogenicity of aflatoxins but also the high sensitivity of certain strains of rainbow trout to chemical carcinogens in general.

Recently, investigators have focused on establishing the sensitivities of certain species of small aquarium fishes to known chemical carcinogens. Stanton (*2*) was the first to use a small aquarium fish, the zebra fish, *Brachydanio rerio,* in this type of experiment, and he found that hepatic neoplasms could be induced in as short a time as 8 weeks after DENA was added to the aquarium water. Later, hepatocellular carcinomas were induced by various carcinogens in 2 more species of small aquarium fish: the guppy, *Poecilia reticulata,* and the medaka, *Oryzias latipes* (*3–6*). In reviewing numerous studies involving the effects of chemical carcinogens on small aquarium fish, Matsushima and Sugimura (*7*) concluded that their use has certain advantages: 1) Fish are typically more sensitive than rodents to the carcinogenic action of most test chemicals. 2) Fish are usually more resistant to the toxic effects of test chemicals. 3) Studies may be conducted in an ordinary laboratory with few special facilities. 4) Larger numbers of fish are more easily maintained than are mammals. 5) Less space and money are used per experiment than if mammals are used. 6) Latent periods for tumor induction are generally shorter in fish than in mammals. 7) Fish systems are more closely related to mammalian systems than are other short-term bioassay test systems (e.g., microbial or *Drosophila*); therefore, results are more applicable to the vertebrate situation.

A small estuarine fish, the cyprinodontid *Rivulus marmoratus* (common name = rivulus), possesses several attributes in addition to those enumerated above which qualifies it as a potential candidate for carcinogenicity testing. It is the only fish known that exhibits natural synchronous hermaphroditism with internal self-fertilization (*8*). This ultimate mode of inbreeding apparently has made these fishes homozygous, and 3 isogenic clones (designated NA, DS, and M) have been identified by intraclonal, interclonal, and interclonal hybrid offspring tissue transplants (*9, 10*). These clones were carefully maintained in the laboratory of Dr. Robert W. Harrington, Jr. for nearly 15 years until his death in 1975. Most of the biological information known about this remarkable species is due to the meticulous efforts of Dr. Harrington (*8–19*).

A rivulus is small (20–50 mm at maturity), easily maintained in the laboratory, has wide salinity tolerances, and oviposits an average of about 20–40 eggs/month

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ABBREVIATIONS: DENA = diethylnitrosamine: ppt = parts per thousand; H & E = hematoxylin and eosin.

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[7] Microslides of liver tumors found in *Rivulus marmoratus,* RTLA 2429-2438, are in the permanent collection of the Registry of Tumors, Smithsonian Institution, Washington, D.C.

(during daylight hours, modally at noon). Generation time is about 6 months. Eggs are large (1.5–2.0 mm diameter) with a transparent chorion. Embryonic development is of sufficient length (12–15 days at 25° C) to allow one to make careful observations on developmental rate and sequence.

The natural habitat of rivulus is shallow estuarine mangrove marshes. Although the species is widely distributed in the Bahamas and Caribbean, it is rare (considered a threatened species in Florida) in the continental United States, possibly due to draining or other alteration of suitable mangrove habitats.

Two types of males exist: primary males, in which ovarian development never takes place, and secondary males, which develop from hermaphrodites (ovarian degeneration). No females have been found in nature. Primary males may be induced by submission of the terminal stages of embryonic development to low temperatures. Secondary males may result from hermaphrodites spontaneously or from exposure of hermaphrodites to various environmental factors (*15*).

Our purpose was to investigate the potential of rivulus as an experimental animal in carcinogenicity testing. Related objectives include the development of simplified culture methods and the establishment of relevant biological data while the fish are held under laboratory conditions.

MATERIALS AND METHODS

Laboratory culture.—The following culture methods have been found optimum for reproduction, development, and growth of the rivulus. Adults are held singly in 4.5-inch (11.4 cm) diameter stackable culture dishes, without aeration, filled with 150–200 ml of synthetic seawater (14 ppt). We fed the adults frozen brine shrimp (*Artemia*) three times a week (once a day for maximum growth), taking care not to overfeed. All uneaten food should be removed soon after the feeding period. Culture dishes are washed in hot water and seawater is changed every month. Under a daily feeding regimen, seawater should be changed twice a month.

Larvae and juveniles are held singly in 2-inch (5 cm) diameter stackable culture dishes also filled with 14 ppt synthetic seawater. Young fish (<3 wk) may be held in groups; however, older fish become quite aggressive, which often results in death for most members of a group if refuges are not provided. Larvae and juveniles are fed freshly hatched brine shrimp nauplii three times a week. The brine shrimp culture is prepared by the addition of 2–6 ml of the brine shrimp eggs to a liter of 14 ppt synthetic seawater in a 1-liter glass separatory funnel outfitted with an air stone, hose, and air pump for aeration. After 24 hours of incubation at 25° C, the air stone is removed for several minutes, which causes the nauplii to fall to the bottom and the egg capsules to rise to the surface. Concentrated nauplii may then be tapped off and fed directly to rivulus larvae and juveniles. If concentrated nauplii are allowed to stand, most will die within 20 minutes due to oxygen depletion. However, 1 or 2 drops of live, concentrated nauplii will remain alive in rivulus culture dishes for several days providing a constant supply of food. Young fish should never be fed dead nauplii or nauplii from a culture much older than 24 hours.

Juveniles are transferred to 11.4-cm diameter culture dishes about 2 months after hatching, at which time the diet may be changed from brine shrimp nauplii to frozen adult brine shrimp, or both may be included in the diet. The diet may also be supplemented with mosquito larvae, which is one of their natural foods.

Eggs are incubated in 5-cm diameter stackable culture dishes filled to 1-cm depth with synthetic seawater (14 ppt). During the terminal stage of embryonic development, hatching must often be induced. Otherwise, embryos may delay hatching for considerable periods, and death may result. Hatching can be induced by a daily change in the incubation water to either seawater or naturally *stained* freshwater. Alternatively, eggs may be dechorionated manually with fine stainless steel watchmaker forceps.

One can collect eggs from hermaphrodites by holding them singly in specially designed reproduction chambers (fig. 1). These consist of 11.4-cm culture dishes with Teflon screens (3- to 4-mm openings) suspended off the bottom of each dish by a glass ring (1 cm thick, 10 cm diameter). Teflon screens are tied to the glass rings with a fine monofilament line that effectively confines the fish to the water space above the screen. Most eggs accumulate between the walls of the culture dish and the glass ring; thus rivulus are prevented from eating their eggs. Eggs and any feces or uneaten food should be pipetted from the reproduction chambers two to three times/week. This can be done most efficiently immediately after feeding. Fish in reproduction chambers are fed individually with thawed brine shrimp held in fine tissue forceps and dangled just in front of them. Six or more can be fed at a time if one proceeds from one to another repetitively until the entire group is fed to satiation. Adults also feed readily on live food, such as mosquito larvae or vestigial winged fruit flies. The optimum temperature and photoperiod for reproduction as well as all other phases of the life cycle is 25° C and 14 hours, respectively.

Comparison of egg incubation techniques.—Because many estuarine cyprinodontid fish deposit their eggs so that development takes place in moist air (*20–24*), a comparison of air incubation and water incubation was made. In a previous study (*25*), air incubation was found superior to water incubation for embryos of *Fundulus heteroclitus.* Development was more rapid and more uniform in air-incubated *Fundulus* eggs.

Twelve rivulus were held singly in reproduction chambers (25±0.5° C, 14-hr photoperiod). We collected the eggs daily and used a random number table to select the appropriate culture technique (odd=air, even=water). Water-incubated eggs were held singly in 5-cm culture dishes filled to 1 cm with synthetic seawater (14 ppt). Air-incubated eggs were held singly in 5-cm culture dishes on 3 layers of Whatman No. 2 filter paper, which had been moistened with synthetic seawater (14 ppt). When embryos reached the terminal stage of embryonic development (stage 32), eggs were inundated for 1 hour with freshly collected seawater. If hatching did not occur within 1 hour, incubation conditions were resumed and the procedure

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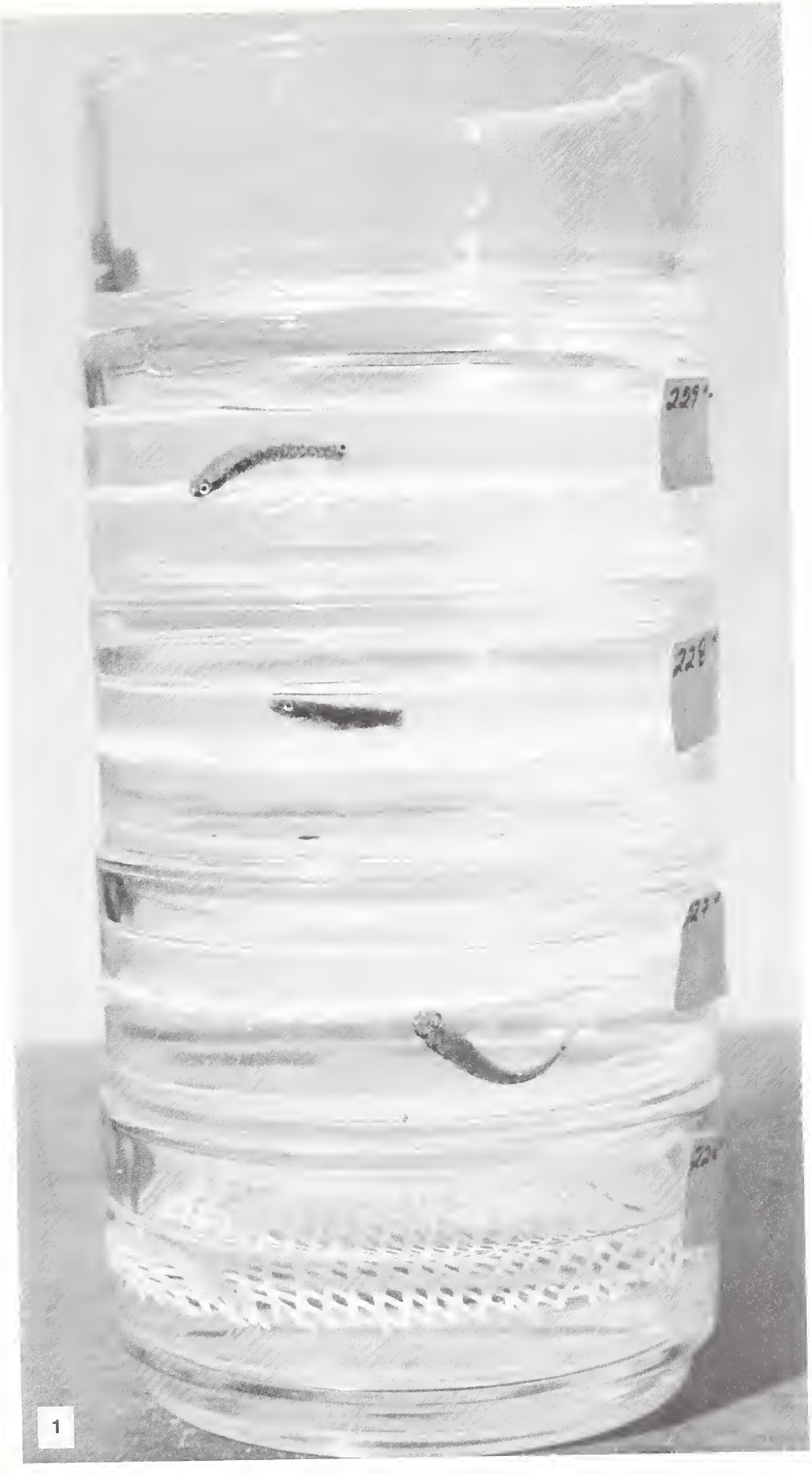

FIGURE 1.—Photograph of reproduction chambers for *R. marmoratus.*

was repeated the following day and so on until hatching occurred. We estimated the approximate age of each embryo by observing its stage of development upon or shortly after oviposition. All embryos were observed and staged daily under low light on a dissecting microscope.

Comparison of the terminal stage of embryonic development (table 1) shows that development in water was more rapid than that in air, although uniformity was approximately the same for both. Therefore, the method chosen for egg development was water incubation.

This culture system has numerous advantages that no other fish culture system has, marine or fresh water. The system is inexpensive, easy to maintain (one technician can easily maintain hundreds of individuals), records may be kept for each individual fish, and large stocks may be kept in a small space. In addition, all culture materials are made of either Teflon or glass, which is essential to avoid contamination by such ubiquitous substances as phthalate ester plasticizers. One disadvantage is that adults must be fed individually when held singly; however, we found that adults may be held in groups if refuges, such as oyster shells, mangrove leaves, glass vials, etc., are provided.

TABLE 1.—*Comparison of rates of embryonic development of* R. marmoratus *in air incubation vs. water incubation*

	Time, hr[a]							
Stage	Air	SE	No. of observations	Water	SE	No. of observations	*t*	*P*
26	115.4	4.66	14	110.5	4.37	11	0.75	NS[b]
27	141.6	5.17	15	135.5	4.61	8	0.77	NS
28	167.3	6.43	8	149.8	4.78	12	2.22	<0.05
29	186.7	4.95	22	176.0	3.97	18	1.88	NS
30	236.3	4.98	27	210.5	5.22	12	3.12	<0.01
31	277.6	7.89	11	241.6	5.59	16	3.84	<0.01
32	291.2	7.67	13	264.8	9.15	12	2.22	<0.05
Hatch	368.6	6.98	13	355.4	7.80	12	1.24	NS

[a] Average time from fertilization to midpoints of stages 26 through 31, and to the beginning of stage 32.

[b] NS = not significant.

Intraparental development.—One result of internal self-fertilization in rivulus is that eggs are emitted at various stages of development. About 1% of the eggs are emitted within 1 hour of fertilization, and about 80% are emitted within 24 hours. The mean intraparental incubation time is about 10 hours or until late blastula stage (*13, 26*). In addition, eggs are frequently oviposited singly; however,

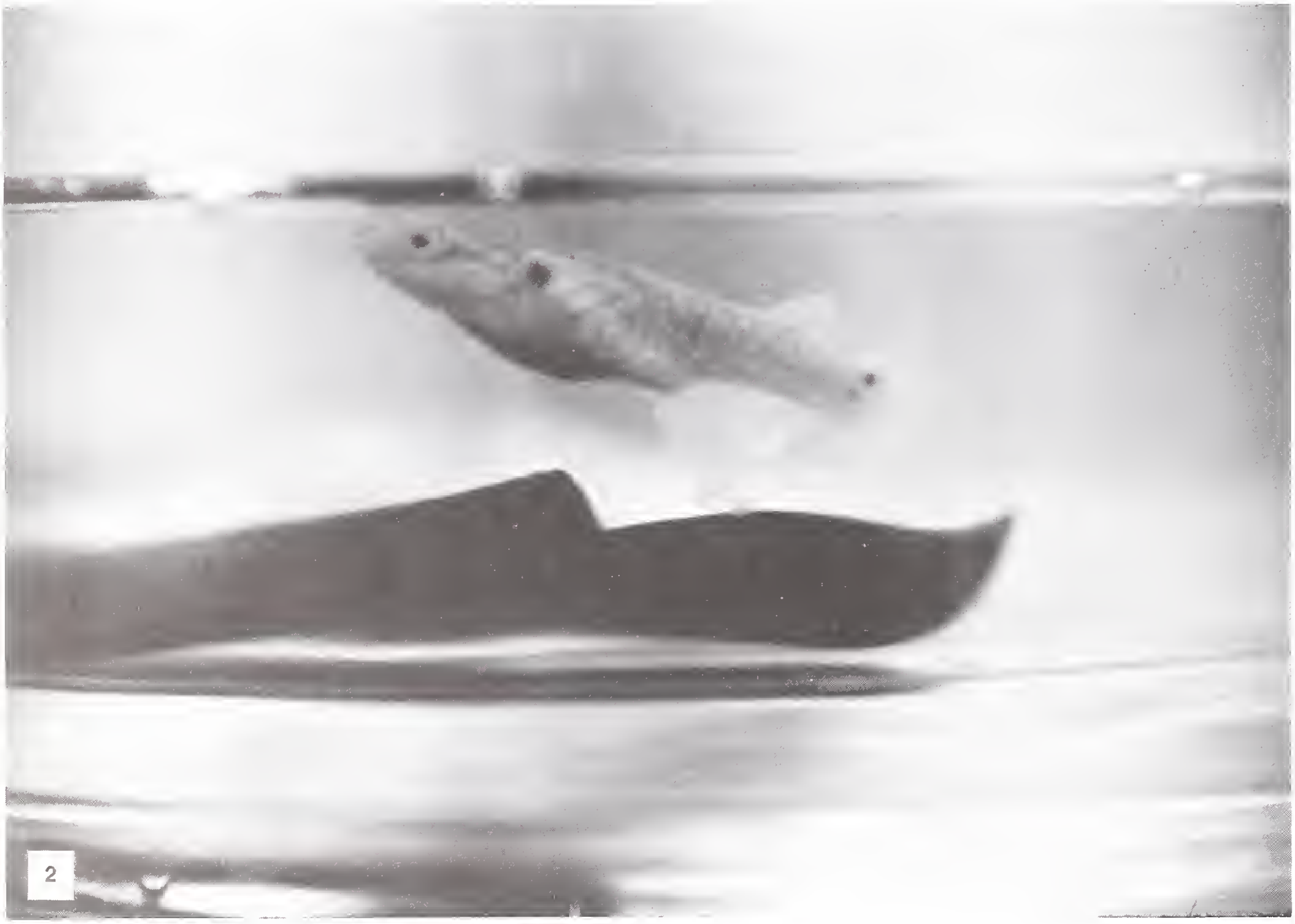

FIGURE 2.—Photograph of *R. marmoratus* with a cluster of eggs.

occasionally clusters of eggs, held together by sticky filaments, are oviposited (fig. 2).

Fecundity.—Fecundity varies among individuals and also with size. Seven healthy adults were observed over a 4-month period. These fish had been acclimated to the conditions in the reproduction chambers for 4 months (November–February) before the 4-month (March–June 1979) observation period. All were healthy proven egg layers that measured 30–35 mm in length: Three belonged to clone NA, 2 to clone DS, and 2 to clone M.

Egg production for all 7 fish averaged 25.3 eggs/month (SE=2.27, $n=28$). Clone NA fish averaged 26.8 eggs/month (SE=3.9, $n=12$), clone DS 20.5 eggs/month (SE=3.8, $n=8$), and clone M produced 27.9 eggs/month (SE=3.8, $n=8$). No statistical difference in egg production could be shown between clones (analysis of variance; F=0.9, $P>0.05$). Overall, 22.5% of the eggs laid were nonviable; most of these were probably unfertilized, although that is not known for certain. Harrington (*13*) carefully observed 6 fish, 4 large (40–50 mm, standard length) and 2 small (25–30 mm, standard length) over a 4-month period (August–November). Eggs were collected immediately upon oviposition. Average egg production overall was 41.5 eggs/fish per month, which is considerably higher than production rates shown here. However, the culture system, food, and feeding regimen were different as well as the average size of the fish. The longer they were held in the reproduction chambers, the more eggs our fish produced. For example, the overall average egg production in March was 11.3 eggs/fish, but the average overall production in June was 38.0 eggs/fish. The overall average egg production of Harrington's (*13*) fish in August was 44.5 eggs/fish, but in November, it was 30.0 eggs/fish. However, his fish were under a natural light regimen. The effect of season on our fish held under constant conditions is unknown. As a rule of thumb, a researcher working with medium-sized, healthy adult rivulus and using the culture system described in this report can expect about 1 egg/fish per day.

Growth.—Optimum container size and relative growth rates were determined photographically following growth in 3 sizes of stackable glass culture dishes. There were 4 replicates for each dish size. Dish diameters were 5.1, 11.4, and 20.3 cm, and all were filled to a depth of 2 cm with synthetic seawater (14 ppt). Lighting was uniformly provided by four 40-watt cool-white fluorescent light bulbs suspended about 1 m above the fish. A standard 14-hour photoperiod and temperature of 25.0±0.5° C were maintained throughout, except for a 24-hour period in February when a power failure caused the temperature to drop to 17° C. Growth rate was recorded by periodic photographing of each fish in its container and next to a metric ruler. At the beginning of the study (4 September 1979), 12 larvae, all hatching on that day and all of clone DS, were randomly assigned to the various containers. All dishes were washed and the water was changed weekly. All fish were fed equal amounts (in random order) of brine shrimp nauplii three times a week for the first 85 days. The quantity of nauplii was adjusted so that some remained at each successive feeding. The young fish could thus feed ad libitum. All fish were switched from brine shrimp nauplii to frozen adult brine shrimp on the 86th day, and they remained on this diet for the next 140 days at which time the study was ended. Frozen brine shrimp were also fed in random order with equal numbers of shrimp to each fish.

TABLE 2.—*Growth of* R. marmoratus *in three container sizes*

Date	Day after hatch	Diameter of dish, cm	Total length, mm	SE	n	Significance[a]
4 September, 1978	0	5.1	5	0	4	NS
	″	11.4	5	0	4	″
	″	20.3	5	0	4	″
21 September, 1978	17	5.1	9.0	0	4	
	″	11.4	9.5	0.29	4	″
	″	20.3	10.1	0.43	4	
27 October, 1978	53	5.1	10.8	0.25	4	
	″	11.4	12.3	0.63	4	$P< 0.05$
	″	20.3	13.3	0.25	4	
28 November, 1978	85	5.1	13.5	0.29	4	
	″	11.4	16.6	0.24	4	$P< 0.001$
	″	20.3	17.3	0.25	4	
24 January, 1979	142	5.1	21.3	0.33	3[b]	
	″	11.4	22.3	0.25	4	NS
	″	20.3	23.7	1.2	3[c]	
27 March, 1979	204	5.1	25.0	—	1[b]	
	″	11.4	26.5	0.29	4	″
	″	20.3	26.3	0.44	3	
17 April, 1979	225	5.1	25.0	—	1	
	″	11.4	26.8	0.25	4	″
	″	20.3	26.7	0.33	3	

[a] Significance was determined by single factor analysis of variance. NS = not significant ($P>0.05$).

[b] Numbers were reduced by death of fish.

[c] One fish jumped out of the container and was lost.

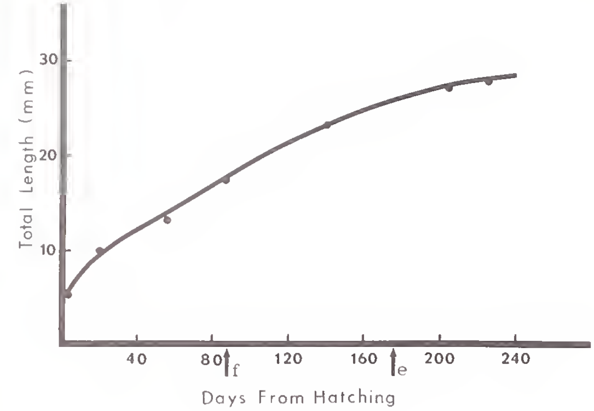

FIGURE 3. Growth of *R. marmoratus* in the laboratory under controlled conditions: 11.4-cm diameter culture dishes, 25.0±0.5° C, 14 ppt salinity, and 14-hr photoperiod. *Arrows* represent time in which diet was changed from live brine shrimp nauplii to frozen adult brine shrimp (f) and time when first egg was laid (e).

Summary data from the experiment are presented in table 2, and the growth rate of fish in the 11.3 cm dishes is depicted in figure 3. The growth rate was rapid during the first 2–3 weeks, then it became almost linear with time until about 160 days at which time it began to stabilize.

Rate of growth was related to the size of dish (table 2). The greatest difference in mean size among the dishes occurred 85 days after hatching and the effects of the smallest dish were greatest. It is possible that feeding nauplii to older fish may have influenced the divergence in size. On the 86th day, the diet was changed to frozen brine shrimp, and all subsequent measurements were not significantly different among the dish sizes (partially due to lost fish).

One fish jumped out of a large dish 28 November 1978 and was lost. All other mortalities occurred in the smallest dishes: 1 died 26 December 1978 (113 days after hatching), 2 died 26 February 1979 (175 days after hatching), and 1 died 17 April 1979 (225 days after hatching). The first egg was laid 28 February 1979 (177 days after hatching) and other fish followed during the subsequent weeks.

Clearly, the optimum size culture dish is 11.4 cm diameter because growth rate was nearly the same as in the largest dishes. Also, the handling and storage of the 11.4-cm dish is much easier. The smallest dish (5.1 cm) is too small for full life-cycle culture but is convenient for eggs, larvae, and juveniles up to about 2 months old. Food type should also be changed from nauplii to frozen brine shrimp at about 2 months after hatching.

Embryonic development.—The description of the embryonic stages of *R. marmoratus* was patterned after Armstrong and Child's (*27*) description of the development of the cyprinodontid fish *F. heteroclitus* and that of Koenig and Livingston (*28*) of the embryonic development of the cyprinodontid fish *Adinia xenica*. Embryonic development is described as occurring in stages. The term "stage" refers to a discrete event or series of events in the developmental sequences. Each stage is centered around an easily recognized event and also includes various minor changes that occur during the same time. All stages are observable with the aid of a dissecting microscope. All times given are midpoints of stages.

Eggs used for determination of stages were obtained after they were naturally emitted from the parental fish. These eggs were collected at least once a day. A minimum of 15 eggs were observed for determination of each stage.

TABLE 3.—*Summary of the development stages of* R. marmoratus *at 25.0±0.5° C, 14.0±0.5 ppt salinity, and 14 hr of light/day*

Stage	Time, hr	Description
1	2.5	1 cell
2	3.5	2 cells
3	4	4 cells
4	4.5	8 cells
5	5.5	16 cells
6	6.5	32 cells
7	8	Early blastula
8	9.5	High blastula
9	10.5	Late or flat blastula
10	15	Gastrulation begins, expanding blastula
11	19	Epiblast covers 1/3 yolk
12	22	Epiblast covers 1/2 yolk, germ ring and embryonic shield are forming
13	24	Epiblast covers 2/3 yolk
14	25.5	Large yolk plug, embryonic shield enlarging
15	31	Epiboly complete
16	34.5	Head and tail regions recognizable
17	36	Optic vesicles appear, somite formation begins
18	43.5	Optocoeles are prominent, auditory vesicles form
19	53	Optic cup and lens formation
20	55.5	Heart beats, no circulation
21	58	Body movement
22	62	Circulation
23	71	Increased vitelline circulation
24	73	Urinary bladder and otoliths first appear, pigmentation on brain, trunk, and yolk near embryo
25	77	Pectoral fins appear, otoliths are prominent
26	90	Liver first appears, pigmentation on optic cup
27	105	Increased pigmentation and body movememt
28	140	Pigmentation of the optic cup obscures the lens, circulation in pectoral fin and liver, caudal fin developing
29	180	Gas bladder and anal fin formation
30	211	Rays in caudal fin form, dorsal fin develops, the jaw appears
31	240	Pectoral fin movement, circulation in the caudal fin, a delay in hatching can occur
32	310	Hatching, rays in the dorsal and anal fin form
33	Post-hatching	Fish is actively swimming

Eggs were handled with flexible forceps or eye droppers and observed on depression microscope slides or in 5.1-cm diameter culture dishes. For ease in observation, the later stages were dechorionated with the use of stainless steel watchmaker forceps.

Observations were made with a Wild M-5 binocular microscope at × 25 magnification with transmitted or reflected light, or both. All photographs of developmental stages were taken at × 10 or 25 magnification with a Nikon camera attached to the microscope.

The following description of the stages of development of *R. marmoratus* under the controlled conditions of 25.0±0.5° C, 14.0±0.5 ppt salinity, and 14 hours of light/day was made by McMillan (*26*). The stages include development from fertilization to posthatching. Stage 1 was observed only once; the description and time from fertilization until the midpoint of stage 1 is from Harrington (*13*). A summary of the stages including the time from fertilization to the midpoint of each stage is given in table 3.

Stage 1: The egg is fertilized and the perivitelline space is present. The blastodisc, which results from the migration of cytoplasm to the animal pole of the egg is also present. The yolk is transparent and contains a mass of oil droplets near the surface. This stage is rarely observed in rivulus because eggs are not released from the parental fish until after internal self-fertilization (fig. 4A).

Stage 2: The first cleavage is meridional, with 1 cell dividing into 2 cells of equal size. Cleavage is meroblastic, thus the yolk mass does not divide. The surface of the egg has a speckled appearance which remains throughout development.

Stage 3: The second cleavage, meridional and at right angles to the first, produces 4 blastomeres of approximately equal size.

Stage 4: The 4 blastomeres divide vertically to form 8 blastomeres.

Stage 5: The blastomeres again divide vertically producing 16 cells, which, when viewed from the animal pole, are in 4 irregular rows of 4 cells each.

Stage 6: Cleavage is horizontal, producing 32 cells arranged in an upper and a lower layer. The cells become progressively smaller as cleavage continues.

Stage 7: The dividing blastomeres have formed a low dome-shaped mass upon the yolk, the early blastula. Blastomeres are piled upon each other, and it is hard for one to distinguish the separate cells.

Stage 8: The high blastula stage is recognizable by the distinct, bulging, bunlike dome of cells on top of the yolk mass (fig. 4B).

Stage 9: During the late or flat blastula stage, the blastomeres are still in a mass, but the margins of the blastoderm are less distinct, and the dome-shaped mass is not as high as previously noted.

Stage 10: The expanding blastula begins gastrulation by epiboly and convergence. The margins of the blastoderm are ragged in appearance.

Stage 11: Gastrulation continues; the cell layer is involved in epiboly; the epiblast covers one-third of the yolk (fig. 4C).

Stage 12: Gastrulation continues; the epiblast covers one-half of the yolk. It is apparent that cells are amassing around the leading edge of migrating blastoderm, the germ ring. Also cells are amassing in one area of the germ ring; the embryonic structures are formed by the two movements of gastrulation, i.e., epiboly and convergence. The mass of large oil droplets is present at the yolk surface near the germ ring.

Stage 13: Gastrulation continues, the embryonic shield enlarges, and the epiblast covers two-thirds of the yolk (fig. 4D).

Stage 14: As gastrulation continues, the yolk material not yet covered by migrating epiblast cells bulges out, forming a large yolk plug. Continued convergence of cells of the germ ring increases the embryonic shield longitudinally.

Stage 15: Gastrulation is complete, and the epiblast encircles the entire yolk. The embryo, lying on the yolk surface, appears to be a furrow running through the mass of oil droplets located directly under the embryo. In oblique view, the embryo appears to be a translucent ridge curving around the surface of the egg.

Stage 16: The embryo continues to lengthen and amass tissue laterally, and the head and tail regions of the embryo are recognizable. In this stage, the embryo is seen as a prominent ridge on the surface of the egg (fig. 4E).

Stage 17: The 3 regions of the brain, the forebrain, the midbrain, and the hindbrain, become visible, and the optic vesicles appear as bulges off the forebrain. Late in this stage, the optocoeles can be distinguished as cavities within the optic vesicles. The first few pairs of somites form, but they are seen with difficulty due to the oil droplets massed below the embryo. The tip of the tail is bulbous, and a mass of tiny droplets is directly posterior to the tail. The pericardial cavity forms and extends on the yolk surface anterior to the head.

Stage 18: The embryo is increasing in size, and somite formation continues. The optocoeles are prominent within the optic vesicles, and the auditory vesicles form lateral to the hindbrain. The pericardial cavity has increased in size and now extends anterior to and lateral to the head. The oil droplets are still beneath the embryo, but the head region now extends beyond the mass of oil droplets.

Stage 19: The optic region is developing with the formation of the optic cup and lens (fig. 4F). The optic cup forms from the optic vesicle by invagination of the lateral surface of the vesicle; thus the optic cup first appears bean-shaped and then cup-shaped. The lens forms within the cup-shaped vesicle. The bulbous tip of the tail is slightly elevated off the yolk. Auditory vesicles are prominent, and the olfactory vesicles are visible. Expansion of the pericardial cavity, now extending from the tip of the head to the first somite, elevates the head. However, the tip of the forebrain is still flush with the yolk. Brain development is increased; the optic lobes appear as lateral enlargements of the midbrain, and a furrow forms down the entire length of the brain.

Stage 20: The heart, visible within the pericardial cavity, extends from the yolk surface anterior to the embryo to the

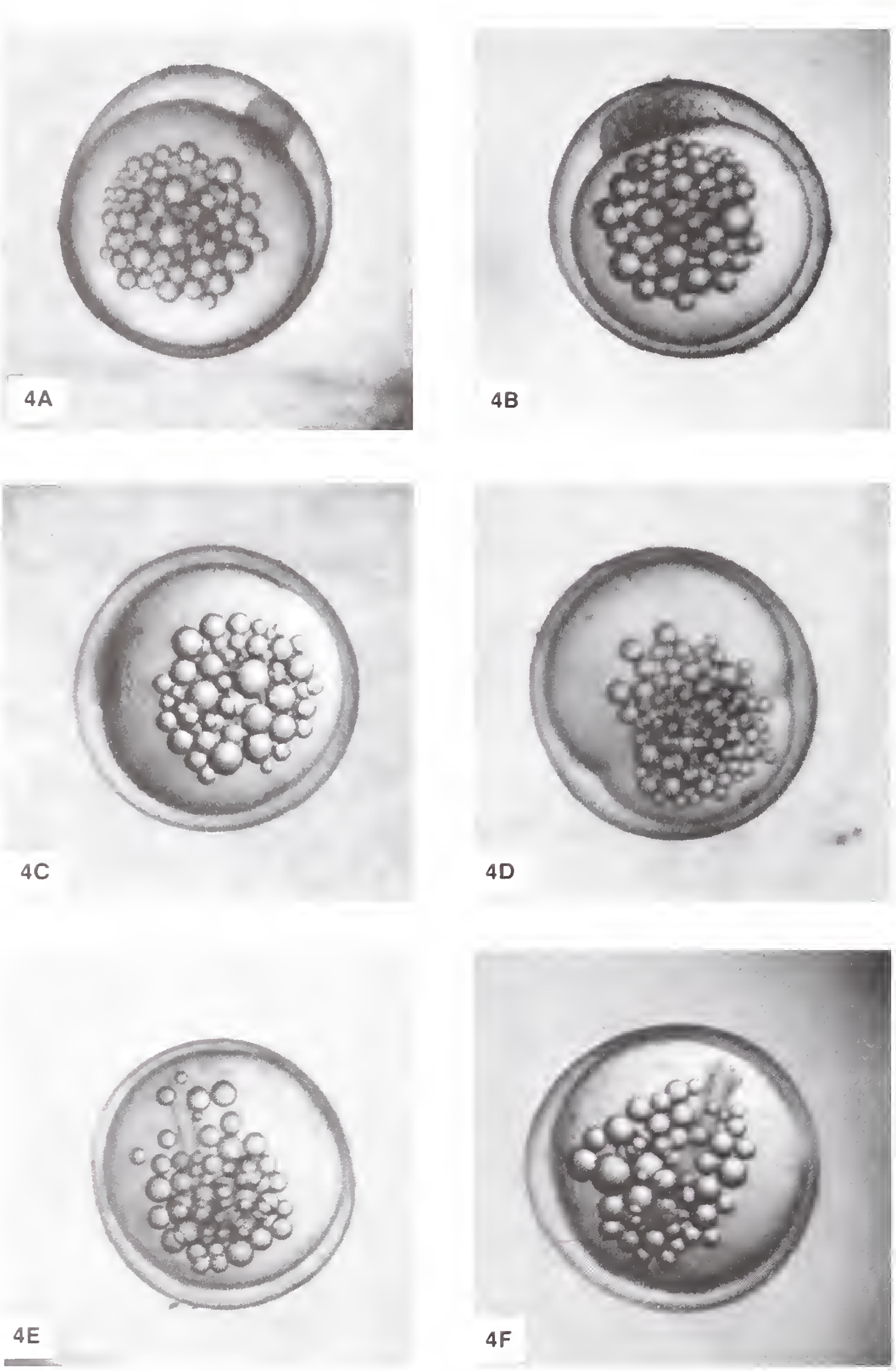

FIGURE 4A–F.—Photographs of some early stages in the embryonic development of *R. marmoratus.* Stages corresponding to figures 4A–F are: 4A, 1; 4B, 8; 4C, 11; 4D, 13; 4E, 16; 4F, 19.

anteroventral surface of the embryo. This tiny tubular heart beats, but no blood circulates.

Stage 21: The anterior somite area has sporadic contractions. Approximately one-half the tail area is free of connection with the yolk, but the tail still lies close to the yolk. A mass of tiny oil droplets is still posterior to the tail. Before circulation, anterior and posterior to the embryo, there is a massing of blood cells.

Stage 22: Circulation begins through the dorsal blood vessel and is observable anterior to the heart and posterior to the junction of the tail and the yolk. The vitelline vessels develop slowly; complete posterior-to-anterior vitelline

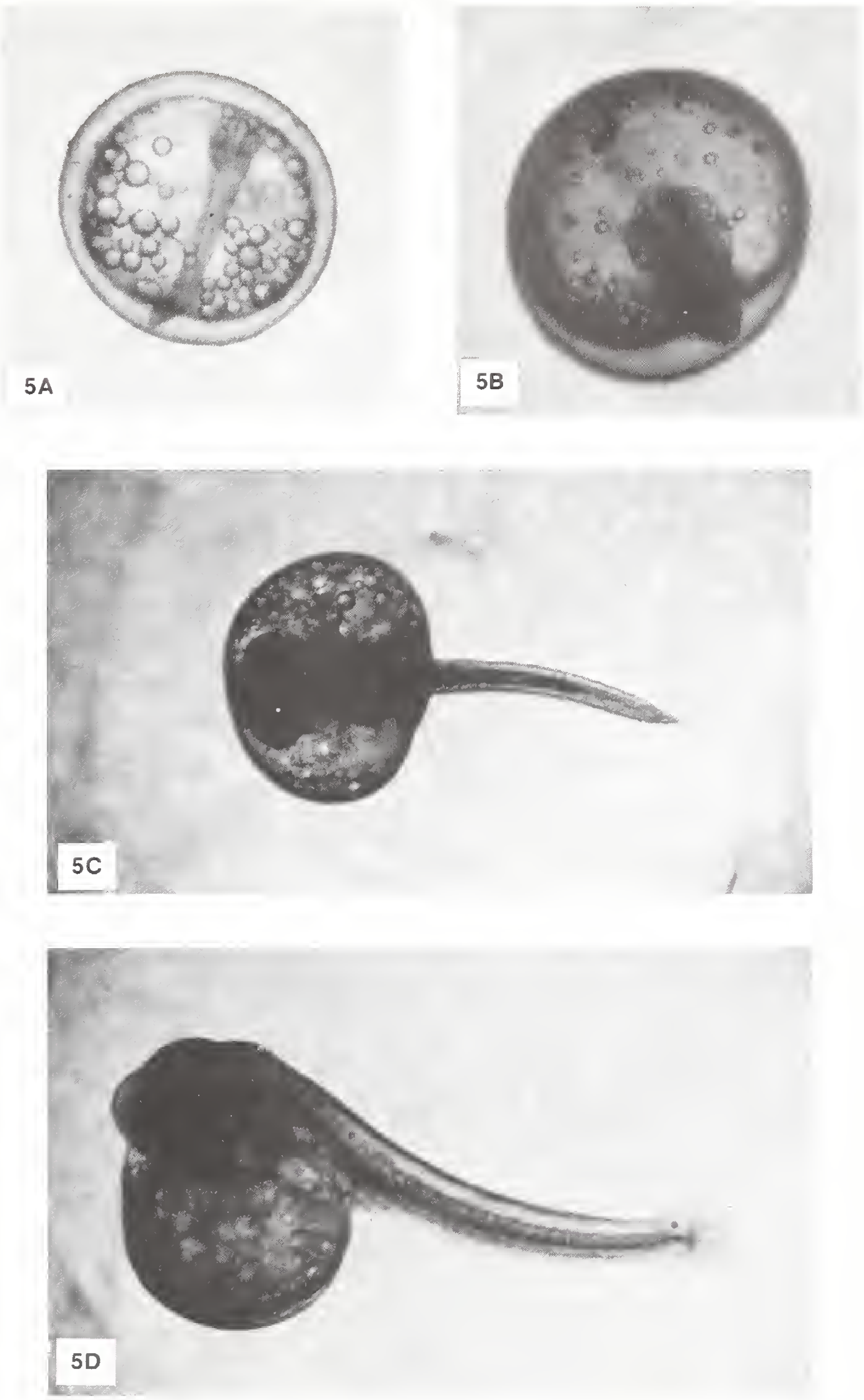

FIGURE 5A–D.—Photographic representations of some late stages in the embryonic development of *R. marmoratus.* Stages corresponding to figures 5A–D are: 5A, 23; 5B, 26; 5C, 28; and 5D, 31. Chorions were removed from embryos 5C and 5D.

circulation is established approximately 2 hours after the onset of circulation. As the vitelline circulation encompasses the entire yolk, the oil droplets, previously clumped together, spread outward until they are evenly dispersed around the yolk. The optic lobes are enlarging, ventricles of the brain are observable, and the brain is taking on a convoluted appearance. A fine network of pigmentation covers the surface of the brain.

Stage 23: Circulation is well established now (fig. 5A). The network of vitelline vessels is increasing; the vessels emerging from the embryo anterior to the somites are especially prominent. The brain continues to enlarge, and the optic lobes become massive and protrude anteriorly, overhanging the posterior portion of the optic cup. Pigmentation covering the embryo increases. The tail occasionally swings sideways.

Stage 24: Organ development increases now that circulation is established. A tiny urinary bladder forms dorsal to the hindgut and ventral to the dorsal blood vessel. The otoliths first appear as tiny specks in the auditory vesicles. Pigmentation on the brain and trunk region increases as well as on the yolk lateral to the embryo.

Stage 25: The otoliths are dense aggregations now, and the pectoral fins appear as tiny triangles protruding from the yolk surface immediately anterior to the first somite.

Stage 26: The pectoral fins lengthen until they project halfway up to the dorsal surface of the trunk (fig. 5B). Pigmentation is generally increased on the head, trunk, and tail of the embryo and on the yolk surface. Pigmentation shades the optic cup a light grey, but the cup is still transparent. The liver bud forms and can be seen on the left side of the embryo immediately posterior to the pectoral fin. Determination of liver bud formation is based on macroscopical, not histological, observations.

Stage 27: The liver increases in size and becomes globular in appearance. Somatic pigmentation continues to increase, especially down the dorsal surface of the tail, which occasionally swings vigorously enough to touch the head.

Stage 28: Pigmentation of the optic cup is so dark that it obscures the lens, and pigmentation of the peritoneum is obvious (fig. 5C). The body cavity is enlarging, as are the pectoral fins, and circulation is observable in them. The caudal fin is forming, appearing as a transparent membrane around the posterior end of the caudal peduncle.

Stage 29: The body cavity is large, lifting the embryo off the yolk surface, but the grey twisting gut does not fill the body cavity. The gas bladder forms dorsal to the gut, but because of dark pigmentation it is hard for one to distinguish. The pectoral fins project as high as the dorsal surface of the trunk, and the anal fin begins developing. In the tail, circulation is observable between the caudal artery and vein.

Stage 30: The rays form in the caudal fin, and the dorsal fin begins developing. Jaw formation is observable and the developing jaws are elevated off the yolk. Pigmentation is heavy, guanine is located along the vertebrae, on the bases of the pectoral fins, and on the surfaces of the optic cups.

Stage 31: The pectoral fins flutter slightly but have no coordinated movement (fig. 5D). Pigmentation is heavy on the dorsal surfaces of the head, trunk, and tail, on the yolk sac, and on the opercula. Circulation is established in the caudal fin. The anal fin is more pronounced than is the dorsal, but no rays are present in either. A delay in further development sometimes occurs at this stage; the embryo can enter a period during which it shows little or no movement and an irregular heart beat.

Stage 32: The mouth opens and closes with accompanying opercular movements, and the pectoral fins are motile. Rays develop in the dorsal and anal fins, and pigmentation appears along the rays of the caudal and pectoral fins. Hatching occurs in this stage.

Stage 33: The young fish actively swims from the moment of hatching. A small amount of yolk is observable on the ventral surface of the body.

Although most eggs of rivulus hatch within 16 days after fertilization, some eggs may take up to 14 additional days after reaching stage 31 to hatch. Eggs of many other oviparous cyprinodontid fishes also undergo delays in hatching (*20, 28*); this delay is apparently related to the ecology of the species. However, in a laboratory situation, it is often necessary for the investigator to induce hatching either by mechanical means (chorion removal with fine forceps) or by other means (e.g., changing the eggs to freshly collected natural water). Otherwise, a significant proportion of the embryos may die through depletion of yolk reserves.

Carcinogen exposure experiment.—Our experimental procedures differed from the routine methods so as to facilitate handling of the fish and to minimize chances of exposure of laboratory personnel to carcinogenic compounds. Several experiments were devised in which adults (>6 mo), larvae (<1 mo), or embryos were exposed in water to which various concentrations of DENA (Sigma Chemical Co., St. Louis, Mo.) had been added.

In all experiments, fish were assigned to various exposure levels randomly (random number table). Temperature, photoperiod, and salinity were 25±1° C, 14 hours, and 14±1 ppt, respectively. Also, containers were cleaned and solutions were renewed weekly. Fish were fed brine shrimp nauplii or frozen adult brine shrimp three times a week. Control fish were always treated identically as exposed fish but without the inclusion of the carcinogen. All exposures were conducted in a light- and temperature-controlled environator outfitted with an exhaust fan. Contaminated air from the environator was periodically purged by way of the laboratory hood, and all transfers and cleanings were done under the hood. As an added precaution, rubber gloves and a carbon filter gas mask were worn during feeding and cleaning. Any concentrated waste was stored in a stoppered glass jar under the hood and disposed of through the hazardous waste disposal procedure at the Medical University of South Carolina, Charleston, South Carolina. Because of our intent to compare the response of rivulus to other fish exposed similarly, concentrations were chosen on the basis of similar published experiments for DENA (*6*). Exposures continued until fish showed signs of toxic stress. Inasmuch as experimental data on embryos were lacking in the literature, exposure concentrations were therefore designated in a log series up to the approximate lethal concentration. The three experiments are described individually: 1) Adult rivulus (30 fish/concentration) were exposed to DENA in the culture water in concentrations of 0, 15, 30, and 45 mg/liter for 5 weeks, then held in uncontaminated water for 1 year before being killed, examined grossly, and prepared for histological examination. Fish were held in groups of 10 in 20.3-cm diameter culture dishes which were kept covered in the environator. 2) Larval rivulus (30 fish/concentration) were exposed to DENA in the culture water in concentrations of 0, 15, 30, and 45 mg/liter for 12 weeks, then held in uncontaminated water for 1 year before being killed, examined, and prepared for histological examination. Each group of 30 larvae were held together in 20.3-cm diameter culture dishes that were kept covered in the environator. 3) Embryos of rivulus (56/concentration) were exposed to DENA in the culture water in concentrations of 0, 10, 32, 100, 320, and 1,000 mg/liter for 1 week. The resulting hatchlings

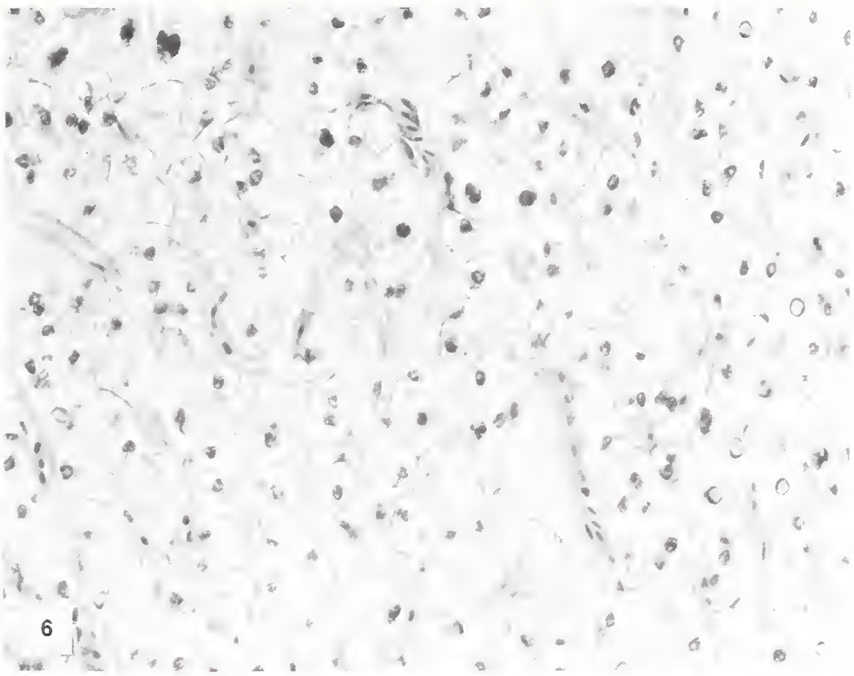

FIGURE 6.—Section of liver from control fish. *Note* light staining and small uniform size of nuclei. H & E. × 400

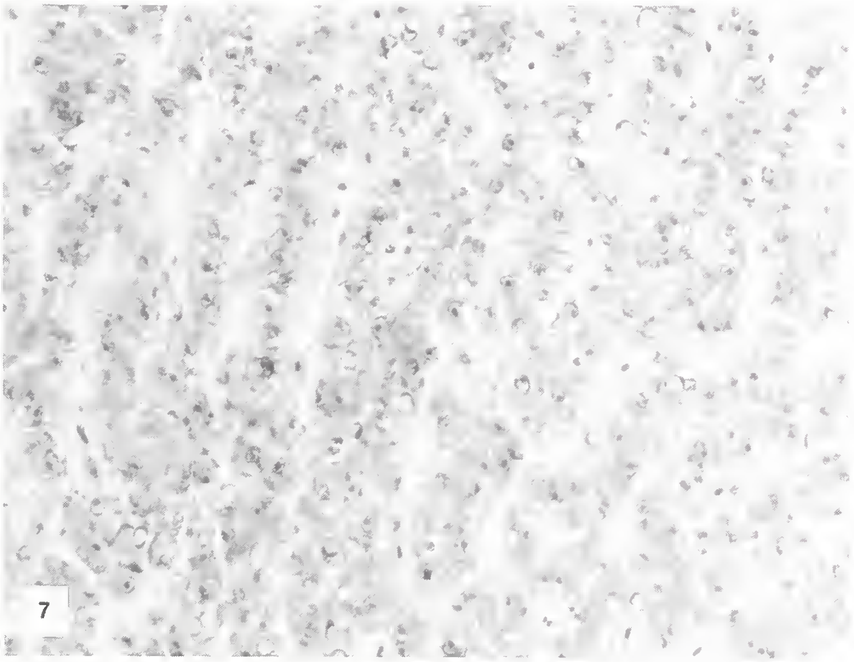

FIGURE 7.—Section of liver from control fish. *Note* dark staining and small uniform size of nuclei. H & E. × 400

were then held for approximately 1 year in uncontaminated water before being killed, examined, and prepared for histological examination. Before exposure, embryos were staged and assigned to 1 of 5 groups: 1) stages 1–9, 2) stages 10–14, 3) stages 15–17, 4) stages 18–23, 5) stages 24–31. (*See* Embryonic Development section.) During all exposures, not more than 5 embryos were held in each 20-ml screw-top test tube sealed with Teflon liners in the environator.

The major emphasis in this study was induction of liver neoplasms. Therefore, fish were killed in 10% buffered formalin solution, and the livers were dissected and processed according to standard histological procedures. Only fish that survived to the end of the experiment were included in the analysis. All livers and occasionally whole bodies were sectioned at several levels, and sections (5–6 μm) were then stained with H & E. Slides were examined and tumors described by J. C. Harshbarger (Registry of Tumors in Lower Animals, Smithsonian Institution, Washington, D. C.) and confirmed by A. J. Garvin (Medical University of South Carolina, Charleston, S. C.).

A single random section through all livers was used in the determination of tumor incidence. It was assumed that the source of error with this method (as opposed to serial sections) was small compared with experimental error.

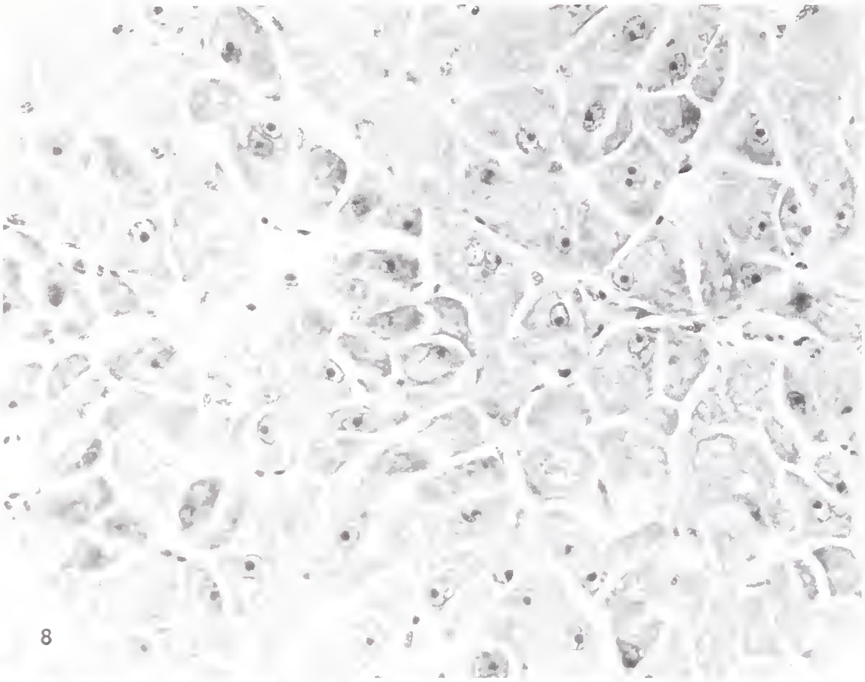

FIGURE 8.—Section of liver from fish exposed to 30 ppm DENA for 12 wk showing cells of a solid pattern hepatocellular carcinoma. *Note* enlarged cells and nuclei with prominent nucleoli. H & E. × 400

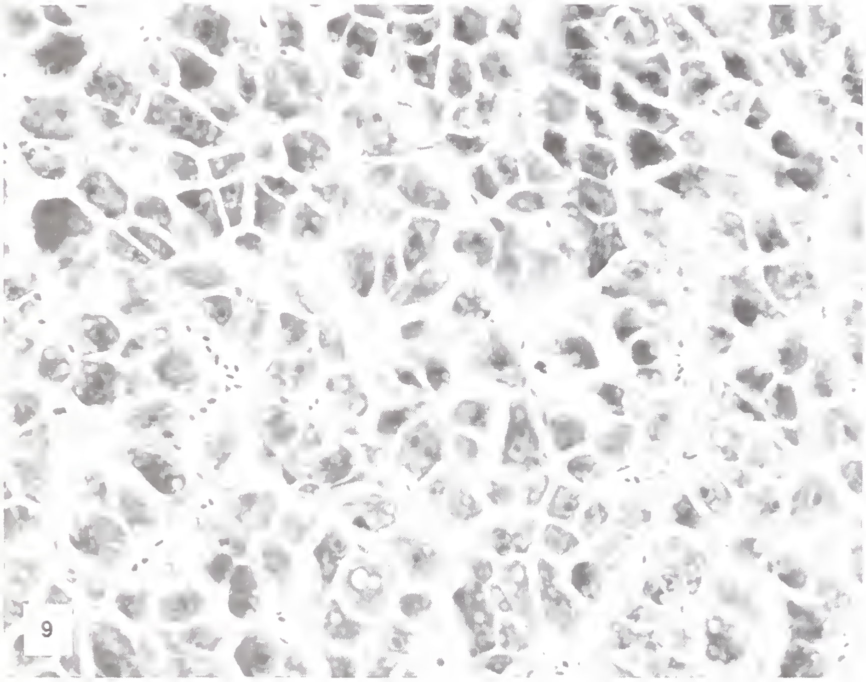

FIGURE 9.—Section of liver from fish exposed to 30 ppm DENA for 5 wk showing cells of a deep-staining, trabecular pattern hepatocellular carcinoma. H & E. × 400

RESULTS

Histologically, the livers of rivulus resemble those of other bony fishes (figs. 6, 7). Liver cords are 2 cells thick, rather than 1 as in mammals, and pancreatic tissue occurs within the liver along vessels rather than as a separate organ. No pathological changes were observed in the livers of the control fish.

In DENA-treated fish from the larval and adult exposure experiments, neoplastic growths originated from both hepatocytes and cholangiocytes. Hepatocellular neoplasms appeared mostly as basophilic nodules, but eosinophilic nodules were also present. Neoplastic nodules showed several patterns: solid (fig. 8), trabecular (fig. 9), and glandular (fig. 10). Different patterns occasionally occurred together in the same liver. Boundaries between tumor nodules and normal liver were distinct sometimes (fig. 11), but no thick fibrous capsules were seen. Other nodules lacked a distinct boundary with tongues of tumor extending into the normal tissue (fig. 12). In the exposure of adult fish, patterns of hepatocellular carcinoma were of all 3 types and of about equal proportions; however, solid and glandular patterns were much more common than the trabecular pattern in the larvae.

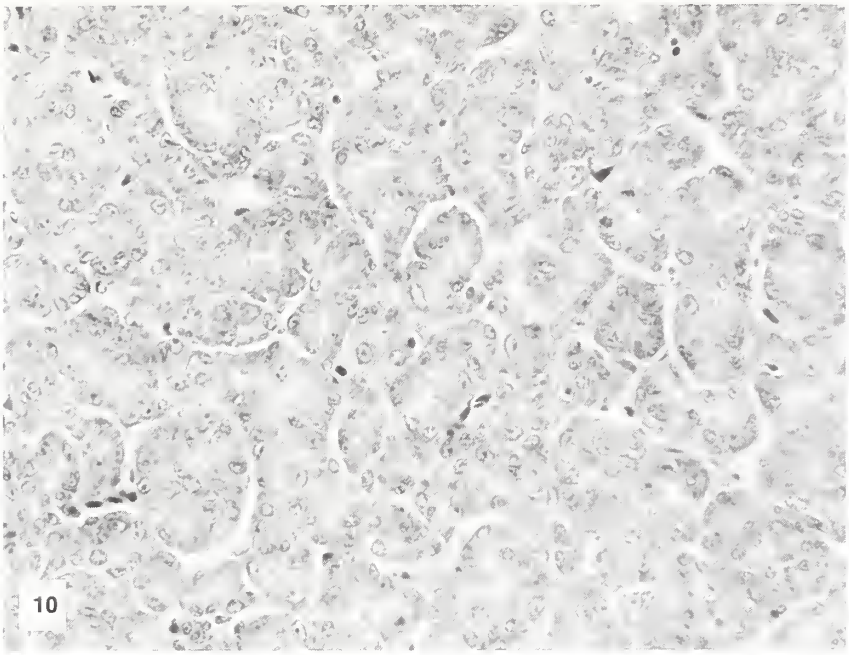
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FIGURE 10.—Section of liver from fish exposed to 15 ppm DENA for 12 wk showing glandular pattern hepatocellular carcinoma. H & E. × 400

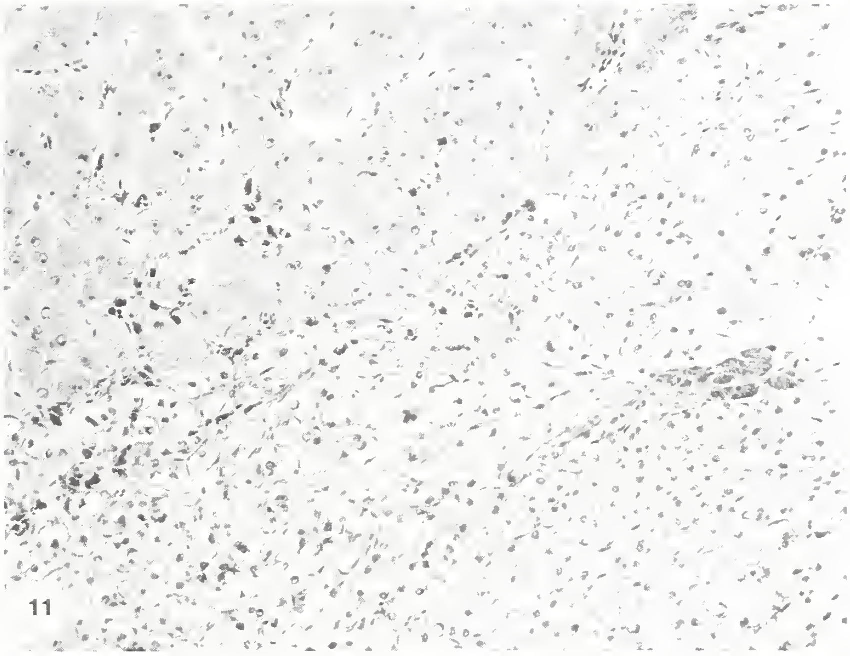
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FIGURE 11.—Section of liver from fish exposed to 30 ppm DENA for 12 wk. *Note* distinct border of solid pattern tumor (*above left*) and normal liver tissue. H & E. × 200

Tumor cells were enlarged as were the nuclei, and nucleoli were distinct. Size varied from only slightly to considerably larger than normal. Most tumor cells were fairly to well differentiated except, in one fish, many tumor cells were spindle-shaped (fig. 13). No tumor cells were so poorly differentiated that all resemblance to normal liver was absent.

Two types of ductular lesions were seen: Cholangiomas (fig. 14) consisted of multiple, thick-walled, various-sized, and randomly oriented ducts or tubules formed by a single layer of tall columnar epithelium. In some ducts, epithelial papillae extended into the lumina. Fibrous stroma was minimal. The other type of ductular lesion had multiple, thin-walled ducts or tubules formed by simple cuboidal or squamous epithelium (fig. 15). They tended to have larger ducts surrounded by smaller satellite ducts. The satellite ducts are considered metaplastic duct formations from the liver parenchyma rather than bile duct proliferations. Most ducts were ensheathed by fibrous tissue, which sometimes was abundant in the interductular spaces. These lesions were interpreted as adenofibrosis. Ductular formations within both types of lesions, cholangiomas and adenofibrosis, contained occasional concretions of unknown composition.

FIGURE 12.—Section of liver from fish exposed to 45 ppm DENA for 12 wk. *Note* indistinct border between tumor and surrounding normal tissue. H & E. × 200

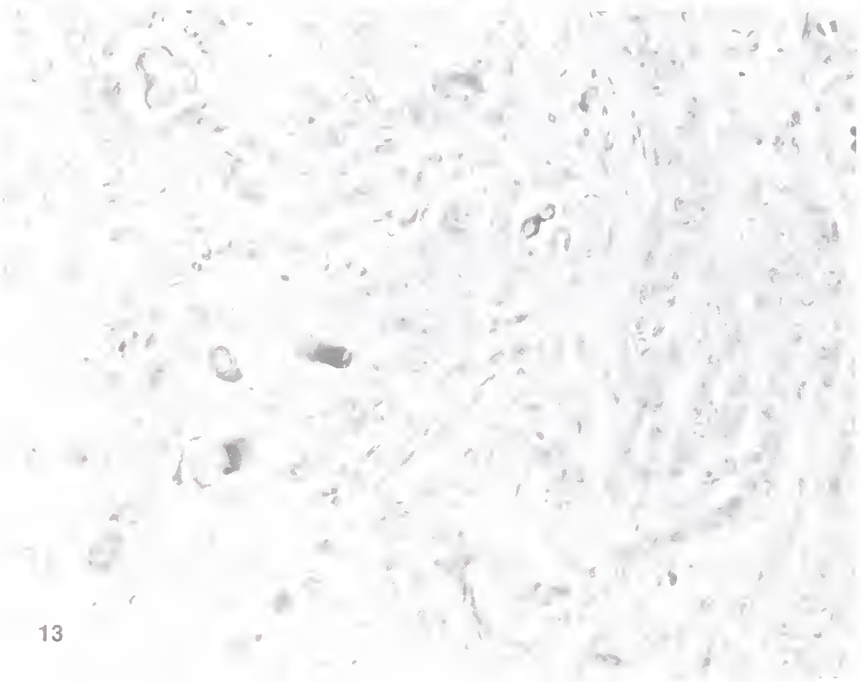

FIGURE 13.—Section of liver from fish exposed to 15 ppm DENA for 12 wk showing spindle-shaped cells of a solid pattern hepatocellular carcinoma. H & E. × 400

Chronic inflammatory lesions included granulomas (fig. 16), comprised of focal collections of epithelioid leukocytes with a fibrous sheath. In some livers, chronic inflammatory lesions, diffuse rather than focal as in granuloma, often contained larger numbers of eosinophils. Foamy macrophages containing a golden pigment were also fairly common.

Dose response among the various pathological changes was not clear; however, the incidence of hepatocellular carcinoma generally increased with exposure level for both larvae (table 4) and adults (table 5). Generally, in the experiment on the 2 highest exposure levels of the larvae, tumors were large and occupied nearly all of the liver on the sections. Also, the numbers of tumors per liver section generally increased with dose level. A threshold of effective concentration is apparent between 15 and 30 ppm for adults exposed to DENA (table 5).

No pathological changes were observed in the livers of fish exposed to DENA as embryos, nor were neoplastic changes found in organs other than the liver, although a complete examination has not been done. Tumors were not apparent on gross examination, but livers of affected fish were generally enlarged and pale in color.

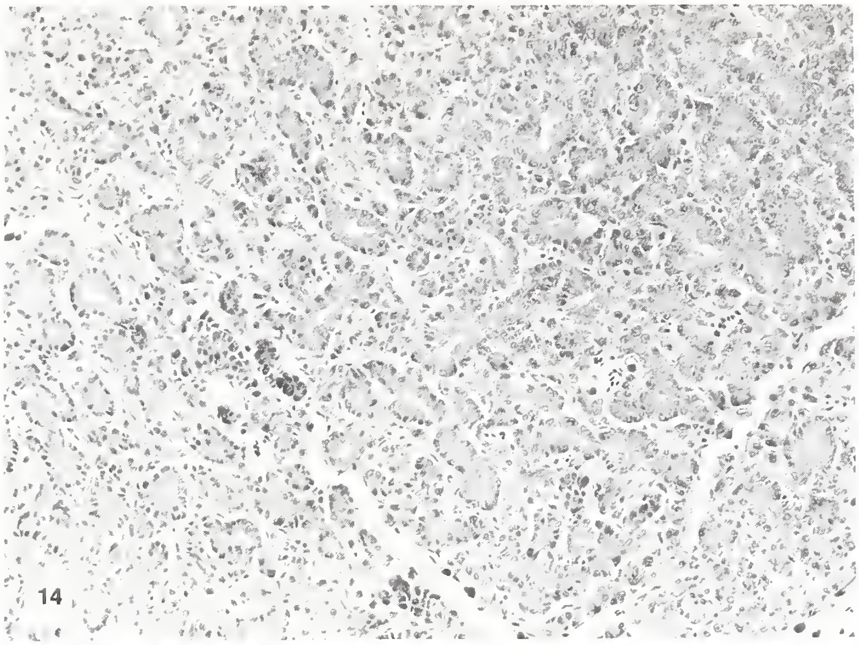

FIGURE 14.—Section of liver from fish exposed to 15 ppm DENA for 12 wk showing bile duct proliferation interpreted as cholangioma. H & E. × 200

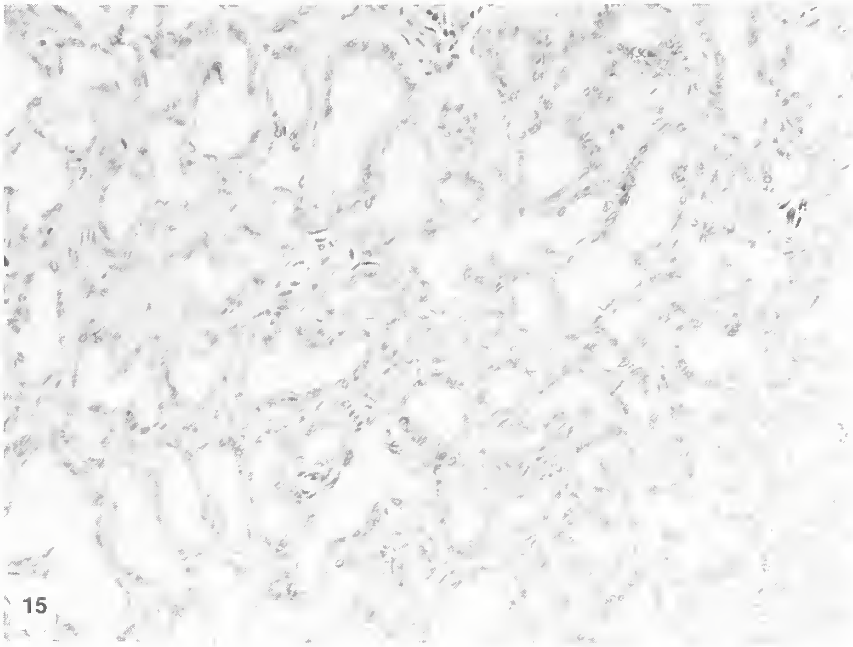

FIGURE 15.—Section of liver of rivulus exposed to 30 ppm DENA for 5 wk showing bile duct proliferation interpreted as adenofibrosis. H & E. × 200

TABLE 4.—*Pathological changes in livers of* R. marmoratus *larvae exposed to DENA for 12 wk*

Nominal water concentrations, mg/liter	No. of fish at:		Incidence[c]											
			Fish with neoplasms[b]		Hepatocellular carcinoma		Incipient neoplasms		Cholangioma		Adenofibrosis		Granuloma	
	Start	End[a]	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
0	30	23	0/23	0	0/23	0	0/23	0	0/23	0	0/23	0	0/23	0
15	30	18	17/18	94.4	12/18	66.7	7/18	38.9	4/18	22.2	16/18	88.9	11/18	61.1
30	30	14	12/14	85.7	9/14	64.3	4/14	28.6	1/14	7.1	6/14	42.9	7/14	50.0
45	30	17	16/17	94.1	13/17	76.5	4/17	23.5	7/17	41.1	14/17	82.4	7/17	41.2

[a] Fish were killed 1 yr after end of exposure period.

[b] Values indicate No. of fish with neoplasms/total No. of fish.

[c] Values indicate No. of fish with pathological changes/total No. of fish.

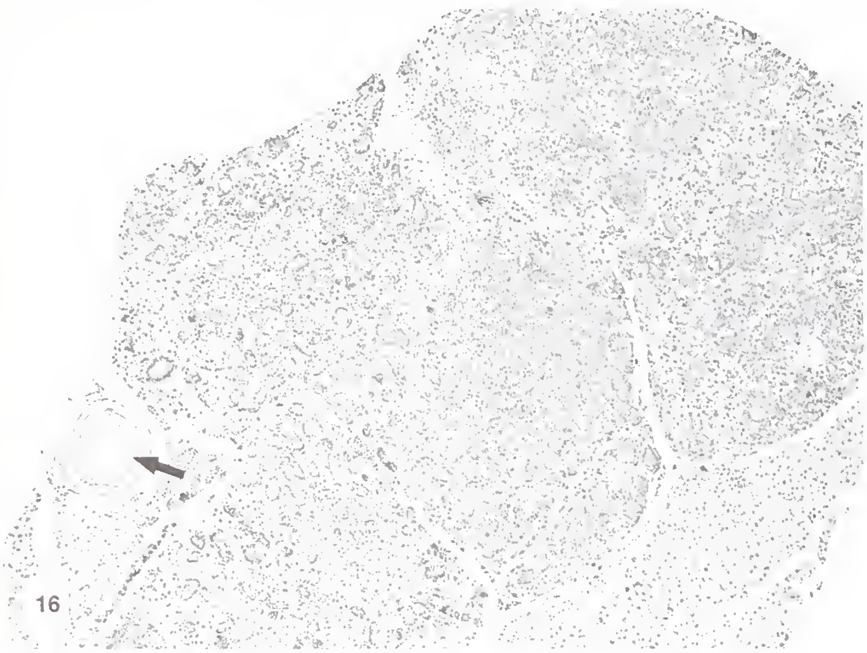

FIGURE 16.—Section of liver of rivulus exposed to DENA for 12 wk showing an encapsulated granuloma (*arrow*), cholangiomatic proliferation (*center*), and glandular pattern hepatocellular carcinoma (*upper right*). H & E. × 100

TABLE 5.—*Pathological changes in livers of* R. marmoratus *adults exposed to DENA for 5 wk*

Nominal water concentrations, mg/liter	No. of fish at:		Fish with neoplasms[b]		Incidence[c]									
					Hepatocellular carcinoma		Incipient neoplasms		Cholangioma		Adenofibrosis		Granuloma	
	Start	End[a]	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
0	30	23	0/23	0	0/23	0	0/23	0	0/23	0	0/23	0	0/23	0
15	30	20	0/20	0	0/20	0	0/20	0	0/20	0	1/20	5.0	0/20	0
30	30	21	7/21	33.3	6/21	28.6	2/21	9.5	1/21	4.8	13/21	61.9	5/21	23.8
45	30	16	5/16	31.3	5/16	31.3	0/16	0	0/16	0	8/16	50.0	2/16	12.5

[a] Fish were killed 1 yr after end of exposure period.

[b] Values indicate No. of fish with neoplasms/total No. of fish.

[c] Values indicate No. of fish with pathological changes/total No. of fish.

DISCUSSION

This study clearly shows that DENA, a potent liver carcinogen in experimental mammals and fish, induces hepatocellular carcinoma among other pathological changes in the liver of rivulus. Because limited numbers of animals were available for this study, termination of the experiment was not staggered. We chose a latent period of 1 year to avoid the ambiguity involved in the characterization of early neoplastic foci. Therefore, it is not known whether a clear determination of hepatocellular carcinoma could be made with a shorter induction period.

The lack of hepatocellular carcinoma in rivulus embryos treated with DENA is difficult for us to explain because some fish embryos (rainbow trout) are considered more sensitive to hepatocarcinogens than are larvae or adults. For example, Wales et al. (*29*) induced liver cancer in a rainbow trout by exposing a series of developmental stages of fertilized eggs to aflatoxin B_1 (0.5 ppm) solutions for 1 hour. The tumor incidence, determined 1 year after exposure, increased as the developmental stage of exposure increased, with the most dramatic rise in incidence occurring near the stage of liver bud formation. Perhaps the lack of response of rivulus in this study relates to the possible impermeability of fish eggs to DENA, as suggested by Hendricks et al. (*30*). However, in a later experiment, they did induce liver tumors in trout by increasing the concentration of DENA (Hendricks JD: Personal communication).

The response of rivulus to DENA was not nearly as dramatic as that of the medaka, *O. latipes*. Our exposure levels (15, 30, 45 ppm) were the same as Ishikawa and Takayama's (*6*), and the length of exposure and conditions were comparable; however, the medaka in their experiment showed tumors as early as 5–8 weeks after exposure to DENA ended. Tumors were obvious on gross examination after 13 weeks, and the medaka usually died of massive abdominal tumors after 20 weeks.

Although the DENA-induced pathological changes in livers of rivulus are similar to those of other experimental animals (*31*), rivulus has several distinct advantages as a test animal for studies involving carcinogenesis. 1) Genetic uniformity of clones eliminates genetic variability as a source of experimental error, which should give better comparability of data between experiments and investigators (*32*). Because self-fertilization is the natural mode of reproduction, the researcher needs no special techniques to maintain isogenicity. 2) Studies involving tissue transplants are possible. 3) The full life cycle is accomplished in the simplest of culture systems, stackable glass culture dishes. Aeration is unnecessary so volatile chemical carcinogens, such as DENA, are not purged from the system. 4) Eggs are emitted daily throughout the year under appropriate conditions of temperature, day length, and feeding. Thus egg exposure experiments such as those described by Sinnhuber et al. (*33*) can be performed at all times of the year. The disadvantages encountered in the use of rivulus embryos in egg exposure experiments are: 1) Eggs cannot be fertilized by artificial means synchronously, and 2) different durations of intraparental incubation cause a variable occurrence of pregastrulation embryos.

The disadvantage of few eggs per fish per time can be easily overcome by maintenance of a large breeding colony. Although individual feeding appears to be a disadvantage, this can be done rapidly for reproducing individuals. Surplus adults may be held communally if refuges are provided, so that aggression is averted.

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DISCUSSION

A. D. Kligerman: I was wondering if the failure to get tumors in the embryo could have anything to do with the embryo not possessing the necessary enzymes.

C. C. Koenig: The embryos that were exposed were late-stage embryos. They were past stage 26, at which the liver bud formation takes place. Work that has been done with trout (I think it was Dr. Hendricks who exposed trout embryos to either dimethylnitrosamine or diethylnitrosamine) indicated an extremely low rate, if any at all, of neoplasms resulting from embryo exposures to nitrosamines. So apparently they are seeing a similar sort of response. They suggested that it was related to impermeability of the vitelline membrane.

C. J. Dawe: This is a little bit out of order, but because we are discussing Dr. Hendricks' work, I would like to have him comment.

J. D. Hendricks: With regard to nitrosamine exposure, our concern initially was that we exposed them for just for 1 hour. We believe that the reason we did not get a response was that this is a highly water-soluble compound and it was not moving into the lipid-rich eggs. Subsequently, with 24-hour exposures, we get a high incidence of tumors with both dimethylnitrosamine and diethylnitrosamine.

F. Anders: I wonder if you, Dr. Koenig, and Dr. Schultz have not shown us tumors growing from the surface of the fish. What may be the reason?

Koenig: I do not know if I can answer that question. Of course, diethylnitrosamine is a precarcinogen, and it needs metabolic activation. The enzymes for that are in the liver. Presumably, that is where the site of the action would be. Other organs also contain those enzymes in lower levels, but it is certainly not as great as the liver.

Dawe: In some other systems, something does happen in the skin.

A. Woodhead: Do you have primary and secondary males in the laboratory, or do they appear only in the wild?

Koenig: In Florida, only 1 primary male has been found. In Curacao, they are more abundant or at least have been. As I mentioned, low temperatures can induce primary males, and the observation of as many as 40% primary males in Curacao was after a season that was unusually cold.

In Florida, I have collected nearly 200 of these animals, and I have yet to find either a primary or a secondary male; that is the situation in Florida. In other areas, they just simply have not been collected, as far as I know.

J. C. Harshbarger: Are these fish compatible or are they cannibalistic? Do you have to keep them isolated, or do you do that just for convenience?

Koenig: If we keep them in a container, i.e., the adults, the larvae can be kept together. If we keep the adults in a container without anything but water, in other words, no refuge, there will be 1 left after a few weeks. However, if we put small refuges, such as oyster shells or other little glass tubes or similar objects in the container, then we have no problem, and that is what we did in these exposures.

J. A. Couch: In regard to Dr. Anders' question about tumors on the surface or the epidermis, recently Henderson and Rose, working with tiger salamanders, found that they have a fairly high level of activity in the mixed function oxidase system in epidermal cells on the body surface. So, I do not know what information is available on the skin or epidermis of fish, but it might be reasonable to suspect that those cells, too, have some activity. You might not be able to ascribe the nonoccurrence of tumors on the skin merely to the possible absence of the mixed function oxidase system in the skin.

Koenig: As you know, the production of mucus is profuse, and I do not know whether these substances are permeable to mucus in the skin; there may be some relationship.

Dawe: Presumably, though, they reach the skin by way of the bloodstream in any case.

Koenig: That is right.

J. B. Gratzek: I was interested in your beautifully simplistic holding system. How often do you change water?

Koenig: We change water about once a month. That is all that is necessary.

Gratzek: That is incredible. With such a compromised liver pathology, do you have secondary problems with infectious diseases or necropsies from other problems?

Koenig: Well, when we first started, we started using synthetic seawater, Instant Ocean sea salts and distilled water. We started out with natural seawater, and periodically, deaths that we could not control did occur. So, the fish are susceptible to diseases. I used various antibiotics, and nothing seemed to stop them until we just simply sterilized everything and put them back under those conditions.

Gratzek: You do not have an ammonia problem or one with oxygen?

Koenig: As I said, these fish are amphibious, and that will give you a good clue as to their fouling of their water. They simply jump out and stick to the side and begin breathing air. In fact, we developed a behavioral bioassay based upon their ability to detect hydrogen sulfide. Hydrogen sulfide, as you know, in mangrove marshes, is a problem for fishes, because it is highly toxic, and these fish can avoid extremely low levels simply by leaving the water and breathing air. They do the same thing under culture conditions; they actually let you know when their water is fouling. Now I do not mean to imply that you cannot kill them, though it does sound like that, it is not true. Certainly overfeeding will kill them quickly, and we have lost a lot by new technicians thinking that the fish need to eat more than they do. Certainly, if you leave uneaten food in the containers, they will die within a matter of a day. So underfeeding them is best, as with most fish.

M. Sigel: Are these malignant tumors or are they simply irritational or inflammatory changes? I have not heard anyone address the question of transplantation of these tumors to inbred fish. Now both of you have homogeneous or inbred strains. Have you transplanted them? Are they autonomous? Have you seen any metastases? Are any of these tumors malignant? These are points that you raised earlier, Dr. Dawe. You were raising questions about pickled flies, and of course, I cannot say too much about differences of opinion, looking at the slide, but I personally think that perhaps some of the answers could have come from transplantation experiments. I have not heard them addressed, either from our speakers from Charleston or Connecticut.

Dawe: Dr. Koenig, have you done any transplantation?

Koenig: No. We have not been dealing with these fish for long. Dr. Harrington had these animals for many years, and his objective was not related to this at all. These data were just available this summer. All that we were interested in, as the first experiment, was to see whether hepatic tumors could be induced by a known carcinogen. This is certainly an obvious way to go from here.

Dawe: Dr. Schultz, have there been any transplantations in your work?

R. J. Schultz: We developed the techniques for transplanting, and it is working well. We are working on what he has suggested but have not completed those experiments.

Unidentified Speaker: Then what is being said, Dr. Sigel, is that these impressions are based on similarities between the lesions seen here and those that have been recorded for years in mammals and other species, but I think you have raised an important point. If transplantation systems are available, now is the time that they be used.

Usefulness of the Medaka, *Oryzias latipes*, as a Test Animal: DNA Repair Processes in Medaka Exposed to Carcinogens[1, 2]

Takatoshi Ishikawa, Prince Masahito, *and* Shozo Takayama[3, 4]

ABSTRACT—Results of previous studies indicate that the medaka, *Oryzias latipes,* a small aquarium fish found in Japan and neighboring countries, is a suitable animal for use in cancer research. This fish showed a high incidence of liver tumors after a short induction period when kept in water containing diethylnitrosamine. Unscheduled DNA synthesis (UDS) was demonstrated in ganglion cells of the medaka in vivo by autoradiography. For this experiment, part of the bony skull of the medaka was removed surgically, and the living fish was kept in an isotonic solution containing a carcinogen and tritiated thymidine. Its brain was then examined histologically. By this method, UDS was clearly demonstrated over the nuclei of ganglion cells after exposure to a wide variety of chemical carcinogens. The abdominal cavity was opened and treated with carcinogens in the same way as the brain; however, no UDS was detected in liver or intestinal cells. We conducted comparative studies on DNA repair in the brains of 5 species of small fish from widely different families. These 5 species (blind cave fish, Oriental weatherfish, medaka, guppy, and Siamese fighting fish) were exposed to various concentrations of 4-hydroxyaminoquinoline 1-oxide or methyl methanesulfonate. We detected UDS in all 5 species after both treatments, although the extent varied in the different species. — Natl Cancer Inst Monogr 65: 35-43, 1984.

The medaka, *Oryzias latipes* (Temmick and Schlegel) a small aquarium fish native to Japan and neighboring countries, appears to be suitable for use in cancer research because it is highly susceptible to some carcinogens. In this paper, the usefulness of this fish is discussed, and some recent results of Japanese cancer researchers are reviewed. Also, our recent studies on DNA repair are described.

The DNA repair mechanisms have been studied in a wide variety of systems ranging from bacteria to mammalian cells, but little is known about DNA repair in fish cells. Here we report a new technique for quantitative measurement of UDS in fish ganglion cells in vivo and the UDS response to treatment in these cells by various chemical carcinogens. We compared the DNA repair levels in 5 species of small fish and in ganglion and other types of cells.

MEDAKA AS A TEST ANIMAL

To arouse your interest in this fish, we will first tell you about it. The medaka or rice fish (fig. 1) is one of the most well-known fish in Japan because of the following traditional childrens' song, which also tells you its habitat.

Medaka's School

"Medaka's school is in the brook
Watch them quietly!
Watch them quietly!
As they play in happy harmony."

Unfortunately, recent land development in this country has disrupted this fish from its natural habitat. Furthermore, its numbers are decreasing because of the extensive use of agricultural chemicals in rice paddies and the invasion of the mosquitofish, *Gambusia affinis,* which was introduced by scientists from the United States to control mosquitoes.

The medaka, a small egg-laying freshwater fish of the family Oriziatidae, is found in some parts of Asia (Japan, Korea, Formosa, and China). It has a short life cycle, and a high capacity for reproduction. Adult fish are 3.0–3.5 cm long and weigh 0.3–0.5 g. Although the wild type of fish (*1*) found in shoals near the surface of flooded rice paddies, ponds, and brooks are brown, colored variants have been known for more than 100 years (*2*). Orange-red and other colors of variants have been kept by goldfish breeders for their beauty. The orange-red variants are called himedaka. (The prefix "hi" means red or scarlet.) These variants are available in shops that sell goldfish in Japan and are the most commonly used fish for various experiments in laboratories (*2*).

The following characteristics of medaka favor the use of these fish in the laboratory: 1) The medaka is one of the

ABBREVIATIONS: UDS = unscheduled DNA synthesis; DENA = diethylnitrosamine; MAM = methylazoxymethanol; 4NQO = 4-nitroquinoline 1-oxide; MMS = methyl methanesulfonate; H & E = hematoxylin and eosin; 4HAQO = 4-hydroxyaminoquinoline 1-oxide.

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[4] We thank Dr. M. Nakadate, National Institute of Hygienic Science, Tokyo, for the ethylnitrosourea and methylnitrosourea; Dr. K. Umezawa, University of Tokyo, for the *N*-hydroxyfluorenylacetamide; Drs. H. Matsudaira and K. Aoki, National Institute of Radiological Science, Chiba, for providing the 26- and 38-month-old fish; and Dr. D. Longfellow, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Maryland, under whose auspices we received the diastereoisomers and K-region epoxide of benzo[*a*]pyrene. We are also grateful for the technical assistance of Miss K. Kawana.

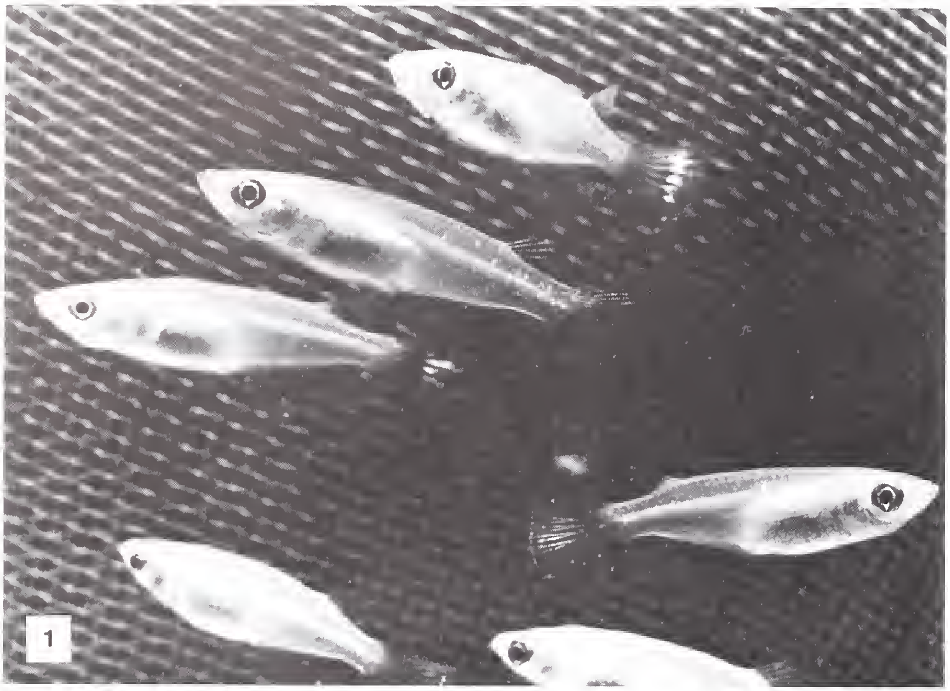

FIGURE 1.—Medaka in an aquarium. This orange-red variety of medaka (himedaka) is commercially available. × 2

smallest vertebrate animals. Therefore, large numbers of individuals can be maintained in a limited space. 2) It is a hardy fish under suitable conditions in the laboratory. It is omnivorous and can be maintained on fresh, dried, or synthetic foods. However, as it is sensitive to the length of daylight, illumination must be carefully controlled; because it lives in still water, recirculation or filtration of the water should be avoided. 3) The medaka is easy to breed under favorable laboratory conditions. A pair of fish lay a cluster of eggs (20–40) almost every morning. Thus embryonated eggs can readily be obtained for experimental purposes. 4) This fish can adapt to a wide range of temperatures (5–30° C) and can be used for studies on the effect of temperature. 5) The secondary sexual characteristics of adult fish are well developed: Males and females are easily distinguishable by the shape of their dorsal and anal fins. 6) Pure strains of medaka have been developed (*3*), and these should be useful in further studies. 7) Egami (*4*) reported on availability of a life-span table of the medaka. 8) Also, cytological data on medaka are available; males and females have 2n=48 chromosomes (*5, 6*).

CANCER RESEARCH

Although the medaka is the most commonly used fish in laboratories for studies in various fields, it had not been used in cancer research until recently. We found that this fish showed a high incidence of liver tumors after a short induction period when DENA was added to the water (*7*). In this work, we followed exactly the procedures used by Stanton (*8*) in his first experiments on small aquarium fish (*Brachydanio rerio*). His first ideas about using "minifish" for cancer research were noted by Dawe (*9*). We found that under certain conditions tumor induction in the medaka was 100% and highly reproducible. Because this fish is highly susceptible to some carcinogens and the time for tumor induction is short, it has also been used in various experiments by other Japanese workers.

Temperature is an important factor in carcinogenesis in poikilotherms. We observed that tumors appeared later and tumor growth was retarded when fish were treated with a carcinogen and maintained at a low temperature (*10*). Kyono et al. (*11, 12*) studied the effect of temperature during and after DENA treatment in detail and concluded that there is an optimal temperature for development and growth of tumors. Kyono and associates (*13*) also reported changes in DNA synthesis and the DNA content of liver cells during DENA-induced tumorigenesis in medaka; partial hepatectomy enhanced tumor formation with DENA (*14*).

Aoki and Matsudaira (*15*) noted the induction of hepatomas in the medaka after treatment with MAM acetate in aqueous solution. Surprisingly, typical trabecular hepatomas were found in 50 to 60% of the fish 60 to 90 days after exposure to 2.0 to 3.0 ppm MAM acetate for only 1 day. This system seems to be especially useful because we have few systems for pulse carcinogenesis in adult rodents. They (*16*) studied the dose-response relationship in this system by treatment with various concentrations of MAM acetate.

Recently, long-term culture of DENA-induced hepatic tumors was achieved by Mitani and Egami (*17*); they subcultured the cells from liver tumors in vitro at 26° C for 18 passages over a period of more than 5 months. These cells grew in a crisscross manner. However, they have not succeeded in establishing cultures of normal liver cells. Egami et al. (*18–21*) reported that the dominant lethal mutation produced by gamma rays or some mutagens in male germ cells of this fish is dose dependent. They suggested that these systems should be useful for the detection of mutagens in an aquatic environment.

DNA REPAIR IN MEDAKA EXPOSED TO CARCINOGENS

Induction of Unscheduled DNA Synthesis in Fish Cerebral Ganglion Cells In Vivo

Ganglion cells of the central nervous system of adult vertebrates are generally considered to be nondividing cells, although in some teleosts new neurons are added to the retina as the eyes increase in size during development (*22*). We (*23*) reported an autoradiographic procedure for detecting UDS in vivo in cerebral ganglion cells of medaka.

Adult medaka (5–7 mo old) were used for this demonstration of UDS. The fish were maintained for 3 days before the operation in the isotonic solution (*24*) prescribed for this fish that was composed of 0.13 *M* sodium chloride, 27 m*M* potassium chloride, 14 m*M* calcium chloride, and 0.3 m*M* sodium bicarbonate. We anesthetized the fish with 0.01% tricaine methanesulfonate (MS-222; Sandoz Pharmaceuticals, Hanover, N.J.) and removed an area (~3 × 3 mm) of the upper part of the bony skull with fine knives and scissors under a dissecting microscope to expose the dorsal surface of the mesencephalon and cerebellum (fig. 2). After this operation, the fish could survive in the

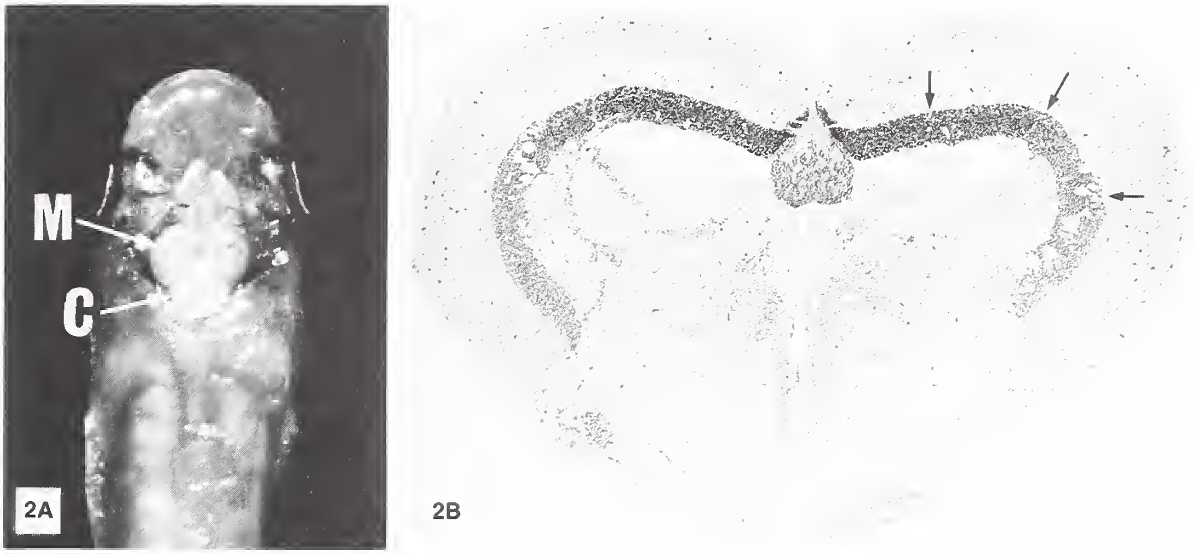

FIGURE 2.—A) Medaka with exposed brain. *Arrows* indicate mesencephalon (M) and cerebellum (C). × 4. B) Transverse section of the mesencephalon showing ganglion cell layer (*arrows*). × 30

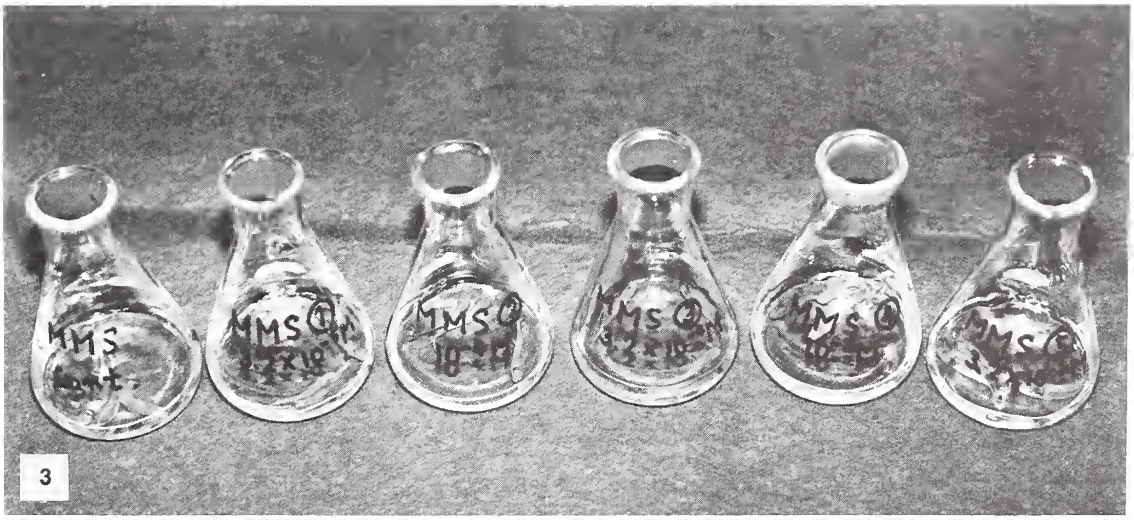

FIGURE 3.—Three blind cave fish with brains exposed are held in a flask at 25° C in isotonic salt solution (20 ml) containing a carcinogen and methyl-[^{3}H]dThd.

isotonic solution for several days without obvious change. After the operation, 3 to 5 fish were transferred to a small incubation flask (fig. 3) containing 10–20 ml of working solution, i.e., isotonic solution containing a carcinogen and methyl-[^{3}H]dThd (New England Nuclear, Boston, Mass.; sp act, 60–80 Ci/mmol) at 10 μCi/ml. In earlier experiments, we tested various concentrations of 3 carcinogens, ethylnitrosourea, 4NQO, and MMS.

We have now tested more than 10 carcinogens in this system (table 1). The incubation time was varied according to the type of carcinogen. In tests with 3 active metabolites of benzo[*a*]pyrene, fish were exposed for 30 minutes to increasing concentrations of these compounds, which were dissolved in a small amount of anhydrous dimethyl sulfoxide and diluted with isotonic solution immediately before use. After exposure, the fish were transferred to a solution containing methyl-[^{3}H]dThd only. After incubation for 3 to 6 hours at 25° C, the fish were fixed in 10% neutral formaldehyde solution. The brain was removed en bloc and cut at the junction between the mesencephalon and olfactory lobe. We tinted the frontal facet of the mesencephalon with red dye (Mercurochrome) to facilitate orientation of the tissue. The brains were embedded in paraffin under a microscope, and frontal sections of 4–5 μm thickness through the mid-mesencephalon were made. Sections were treated with 3% perchloric acid, dip-covered with NR-M$_2$ emulsion (Konisiroku Photo Co., Tokyo, Japan) and developed after exposure for 5 weeks at 4° C. Preparations were lightly stained with H & E. Grains on 100 to 200 randomly selected ganglion cell nuclei in each brain (fig. 2) were counted under a × 100-objective lens (oil immersion) of a Nikon microscope. Similarly, we counted the background grains from ganglion cell nuclei of control specimens. As a result of oblique emission from the nucleus, a few grains were seen around the margin of the nucleus; they were excluded from grain counting.

As shown in representative photomicrographs (fig. 4), UDS was clearly demonstrated as silver grains on the nuclei of ganglion cells of groups treated with carcinogens. The background count was 0.2–0.3 grains/cell in the multiple controls used in each experiment. Only those chemicals that induced significantly higher UDS levels than background were regarded as giving positive results. Almost all ultimate or proximate carcinogens tested induced UDS to varying degrees. *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine and *N*-hydroxyfluorenylacetamide were weakly positive. Of the procarcinogens tested, 4NQO, DENA, and dimethylnitrosamine induced detectable UDS, whereas benzo[*a*]pyrene and aflatoxin B$_1$ did not. These carcinogens may not be metabolized to the active form in ganglion cells. Among the active metabolites of benzo[*a*]pyrene tested, the two diastereoisomers (BPDE I and BPDE II) were equally effective in inducing UDS. However, the K-region epoxide (BPE) gave a weakly positive result in fish ganglion cells. The highest average grain number in carcinogen-treated groups was approximately 16–17 times that of the control. We usually used the acute LD50 (in 24 hr) concentration of each carcinogen as the highest dose. The chemicals that induced UDS had roughly dose-dependent effects.

We used 4 groups of male fish 4 to 38 months old[4] to study the effect of aging on the level of DNA repair. In these groups of 5 to 10 fish each treated with 4NQO and methyl-[^{3}H]dThd, we found no age-associated change in the level of UDS in ganglion cells of the medaka (*23*). The oldest medaka used in this study (38 mo) represented the maximum age of this fish (*4*).

TABLE 1.—*Induction of UDS by various chemicals in ganglion cells of medaka*[a]

Chemical	Dose range, *M*	Maximum No. of grains/cell	Induction of UDS	Induction period, hr
MMS[b]	10^{-4} – 3.2×10^{-3}	2.7[c]	+	4
Methylnitrosourea	10^{-5} – 10^{-3}	3.3[c]	+	3
Ethylnitrosourea[b]	10^{-4} – 3.2×10^{-3}	2.9[c]	+	4
N-Methyl-*N*′-nitro-*N*-nitrosoguanidine	3×10^{-7} – 3×10^{-5}	1.3[d]	+	3
N-hydroxy-2-fluorenylacetamide	3×10^{-7} – 3×10^{-5}	1.5[d]	+	3
4NQO[b]	2.5×10^{-6} – 4×10^{-5}	3.0[c]	+	3
4HAQO	3.2×10^{-5} – 10^{-4}	6.2[c]	+	3
Dimethylnitrosamine	4×10^{-5} – 4×10^{-3}	2.1[c]	+	6
DENA	3×10^{-5} – 4×10^{-3}	2.3[c]	+	6
Benzo[*a*]pyrene	4×10^{-7} – 4×10^{-5}	0.3	−	6
BPDE I	2×10^{-7} – 2×10^{-5}	2.2[c]	+	3.5
BPDE II	2×10^{-7} – 2×10^{-5}	3.5[c]	+	3.5
(+)-4,5-Dihydrobenzo[*a*]pyrene-4,5-epoxide	2×10^{-7} – 2×10^{-5}	1.5[c]	+	3.5
Aflatoxin B$_1$	6×10^{-8} – 6×10^{-6}	0.3	−	6
Caffeine	5×10^{-7} – 5×10^{-5}	0.2	−	3
Control		0.2–0.3		3–6

[a] The following chemicals were purchased from the sources indicated: MMS, Aldrich Chemical Co., Milwaukee, Wisc.; 4NQO and 4 HAQO, Iwai Chemical Co., Tokyo; dimethylnitrosamine, benzo[*a*]pyrene, and DENA, Tokyo Kasei Co., Tokyo; *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, Wako Pure Chemical Co., Tokyo; aflatoxin B$_1$, Makor Chemical Ltd., Jerusalem, Israel; and caffeine, Nakarai Chemical Co., Tokyo. The balance were gifts; *see* footnote 4.

[b] Chemical was tested previously.

[c] $P < 0.02$.

[d] $P < 0.05$ by *t* test.

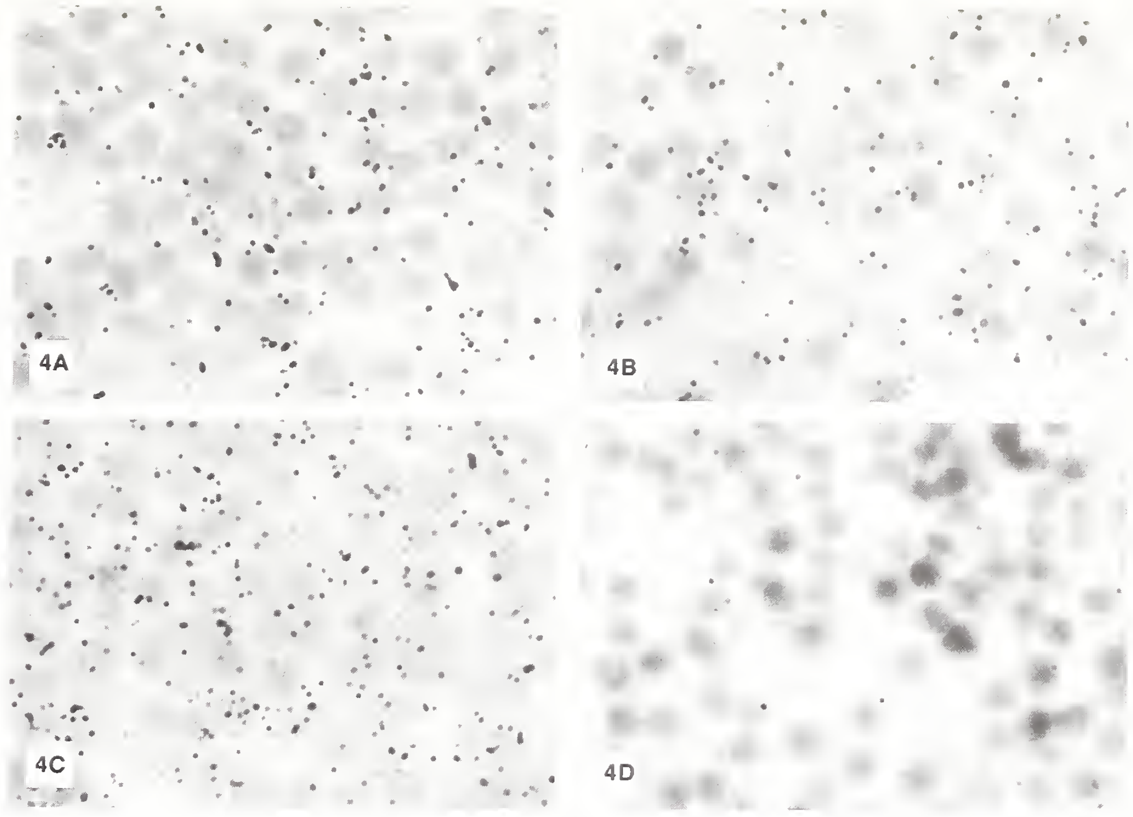

FIGURE 4.—A) Autoradiograph showing silver grains on the nuclei of cerebral ganglion cells indicative of UDS after treatment with 10^{-3} *M* *N*-methyl-*N*-nitrosourea and methyl-[^{3}H]dThd for 3 hr. Lightly stained with H & E. × 1,500. B) Autoradiograph of medaka brain after treatment with 4 × 10^{-3} *M* DENA. H & E. × 1,500. C) Autoradiographs of medaka brain after treatment with 2 × 10^{-5} *M* BPDE II. H & E. × 1,500. D) Control autoradiograph showing few background grains after treatment with methyl-[^{3}H]dThd only. H & E. × 1,500

One advantage of this method is that ganglion cells can be studied in the living state and can be treated efficiently by continuous contact with isotonic external media as in an in vitro study; the brain of this fish is small enough to allow free penetration of the medium. Thus far we have been unable to demonstrate UDS by injecting a chemical carcinogen and methyl-[^{3}H]dThd into an intact fish nor to demonstrate any UDS grains without exposing the brain.

Unscheduled DNA Synthesis in Intestinal Cells and Liver Cells of Medaka In Vivo

To detect UDS in other cells besides those of the ganglion, we applied a similar technique to the abdominal organs. An outline of our procedures is as follows: Under a microscope, we cut an oval fenestration (4×5 mm) in the anterio-median region of the abdominal wall to expose the ventral surface of the liver and intestinal loop. Again, the fish could survive without obvious change for 6 to 8 hours in an isotonic solution. After the operation, each fish was placed in a small flask containing 10 ml of working solution with a carcinogen and methyl-[^{3}H]dThd for 3 hours at 25° C. The following 4 carcinogens were tested at 3 doses: methylnitrosourea (10^{-3}, 10^{-4}, and 10^{-5} *M*); ethylnitrosourea (10^{-3}, 10^{-4}, and 10^{-5} *M*); 4NQO (10^{-5}, 10^{-6}, and 10^{-7} *M*); and MMS (10^{-3}, 10^{-4}, and 10^{-5} *M*). Then the fish were fixed in 10% neutral formaldehyde solution, and their abdominal organs (i.e., liver and intestine) were removed. The tissues were embedded in paraffin and sectioned. The autoradiographic procedure was the same as for the brain. No UDS was detectable in either liver or intestinal cells in response to any of the carcinogens tested.

Species Differences in DNA Repair

Using the fish brain system, we compared DNA repair in 5 species of small fish. The fish we examined, all purchased from an aquarium shop in Tokyo, were as follows: blind cave fish (*Anoptichtys jordani,* 5–6 mo old, both sexes); Oriental weatherfish (*Misgurnus anguillicaudatus,* 3–4 mo old, both sexes); medaka (*O. latipes,* 5–6 mo old, male); guppy (*Poecilia reticulata,* 6–7 mo old, male); and Siamese fighting fish (*Betta splendens,* 6–7 mo old, male); all were considered as young adult fish. We chose these species because they belonged to widely different families, but their brains were similar in size. We obtained information on their age from the aquarium shop. Data on their body weights, brain sizes, etc. are given in table 2. We used essentially the same technique as that for the study on the medaka brain. Because it was essential to compare widely different species under as nearly similar conditions as possible, we used the same vials of carcinogens and methyl-[^{3}H]dThd, completed the series of experiments within 6 days, and performed all autoradiographic procedures simultaneously. However, we varied the volume of working solution (10–30 ml) for 3 fish according to the size of the fish. Grains were counted on 400 to 500 ganglion cells from each fish. We tested the inductions of UDS with 2 types of carcinogens, i.e., 4HAQO (UV type) and MMS (X-ray type). These carcinogens were tested at a logarithmic series of concentrations as shown in figures 5 and 6. Groups of 3 fish of each of the 5 species were incubated in small flasks with 5 to 6 concentrations of each carcinogen for 3 hours at 27° C (fig. 3). All the species except the guppy survived until the end of incubation with the highest concentration of MMS (10^{-3} M). A higher concentration (3.2×10^{-3} M) was toxic to all species. Because some of the guppies died during treatment, data on these fish are not included. One carcinogen, 4HAQO, appeared to be less toxic, and a species exposed to even a saturated solution of it (2×10^{-4} M) survived for at least 24 hours. For convenience, we chose 10^{-4} M as the highest concentration of 4HAQO.

In this type of study, it is important for one to examine possible differences in dThd pools in different species. The optimal concentration of methyl-[^{3}H]dThd was determined as follows: Groups of 3 fish of each of the 5 species were treated with 3 doses (10, 20, and 30 μCi/ml) of methyl-[^{3}H]dThd and 4HAQO (3.2×10^{-5} M). Results showed that the numbers of grains on the ganglion cells increased with the concentration of methyl-[^{3}H]dThd up to 20 μCi/ml and then reached a plateau (data not shown). Therefore, in this experiment, we used methyl-[^{3}H]dThd at a concentration of 20 μCi/ml. Under these conditions, error due to the effect of size difference in the internal dThd pool was probably insignificant.

TABLE 2.—*Species of fish used for comparative study of UDS*

Species	Family	Body weight, g	Width of mesencephalon, mm
Blind cave fish (*A. jordani*)	Characidae	0.35–0.45	2.5–2.6
Oriental weatherfish (*M. anguillicaudatus*)	Cobitidae	0.8 –1.2	2.5–3.5
Medaka (*O. latipes*)	Oryziatidae	0.3 –0.4	2.8–3.2
Guppy (*P. reticulata*)	Poeciliidae	0.35–0.45	2.5–2.8
Siamese fighting fish (*B. splendens*)	Anabantidae	1.0 –1.3	3.0–3.5

Figure 5 shows the dose–response curve of the grain counts in the 4 species of fish treated with MMS. All 4 species showed active UDS in response to MMS, and the levels of UDS response to it did not vary markedly in different species.

The dose–response relationship for UDS in the 5 species treated with 4HAQO are shown in figure 6. Although UDS was detected in all the species tested, its extent varied in different species.

DISCUSSION

Although DNA repair mechanisms have been studied in many different systems ranging from bacteria to mammalian cells (*25*), these surveys included only a few species and provided little evidence of interspecies differences in DNA repair. Moreover, few studies were aimed specifically

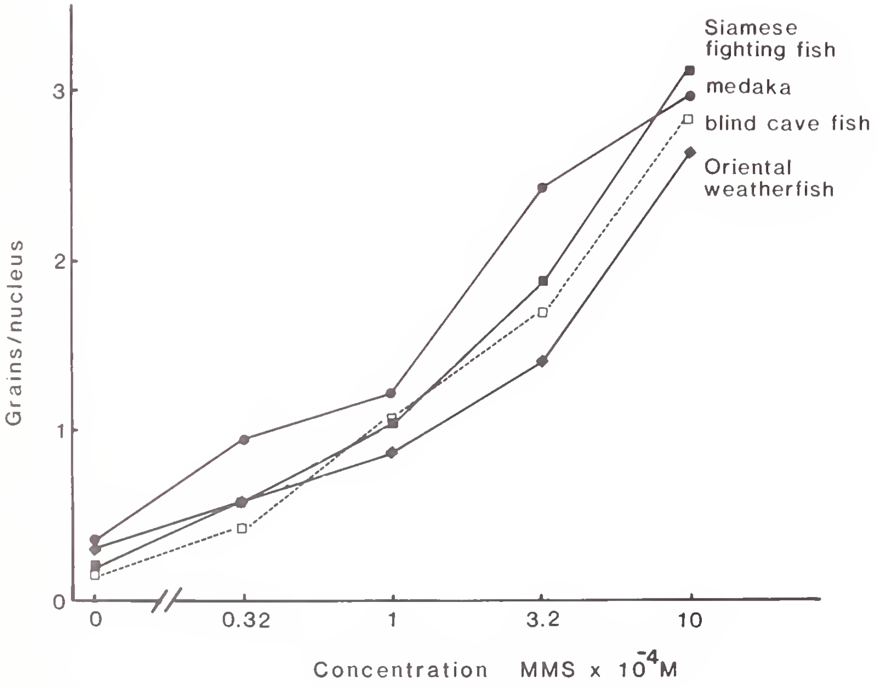

FIGURE 5.—Dose–response relationships for UDS in cerebral ganglion cells of various fish treated with MMS. No. of grains are averages for 3 fish.

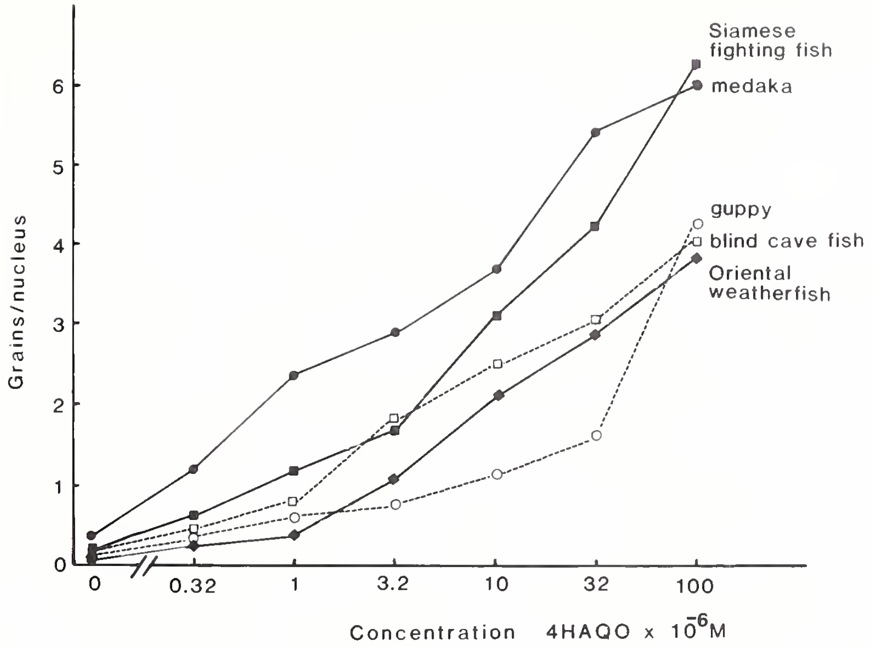

FIGURE 6.—Dose-response relationships for UDS in cerebral ganglion cells of various fish treated with 4HAQO. No. of grains are averages for 3 fish.

at investigations of DNA repair in fish cells. Fish occupy a distinct segment of the evolutionary tree and constitute the largest and most diverse class of vertebrates. With over 20,000 different species, enormous spectra of reproductive mechanisms, sizes, and longevities (*26*) are represented. Therefore, a study of DNA repair in fish should enable us to draw some general conclusions about the universality or specificity of DNA repair mechanisms.

We have shown that many chemical carcinogens induced UDS in fish ganglion cells. Our findings suggest that fish cells can repair different types of DNA modification induced by chemical carcinogens. It is generally believed that carcinogen- or UV light-induced DNA damage can be repaired by at least two mechanisms: excision and postreplication repair (*27–29*). The latter mechanism appears to be important in replicating animal cells. However, in nondividing ganglion cells in which postreplication repair processes are nonoperative, the excision type is probably the mechanism of DNA repair. With our UDS detection methods, carcinogen-treated liver or intestinal cells of medaka do not exhibit UDS in vivo because if repair replication were occurring, dThd should be incorporated into DNA. Achey et al. (*30*) observed that thyroid cells of *P. formosa* have photoreactivation activity, but they do not show any detectable excision repair. Shima and associates (*31*) obtained similar results with cultured cells from goldfish fins. These data are consistent with our observations on liver and intestine of medaka in vivo. However, Stich and Acton (*32*) determined that some cultured lines of fish cells show UDS induction in response to various chemical carcinogens. Fish cells deficient in the excision repair mechanism should be especially susceptible to chemical carcinogens. However, it remains to be established if other mechanisms, such as bypass synthesis or recombination repair, are operative in fish cells. With respect to mammalian cells, Setlow et al. (*33*) reported that excision repair does in fact occur in rodent cells, although at a significantly lower rate than in human cells. Dimer excision in mouse embryo fibroblasts decreased rapidly during progressive passage of the cells in culture (*34*). In this sense, mouse fibroblasts resemble human xeroderma pigmentosum cells. In contrast to these previous reports on fibroblastic cells in culture, Bowden and co-workers (*35*) and Cooke and Johnson (*36*) observed that mouse epidermal cells in vivo are capable of DNA excision repair after UV irradiation. We (*37*) also reported that mouse tracheal epithelial cells and retinal ganglion cells show active UDS induction in organ culture. In another study, we (*38*) found that UDS is far more active in mouse skin epithelial cells than in dermal fibroblastic cells in vivo. These observations suggest that the efficiency of DNA repair depends on both cell type and stage of development.

In studies on photoreactivation, much attention has been directed to fish cells (*30, 31, 39–41*) because they contain a large amount of enzyme. An interesting finding was that this photoreactivation enzyme is present in the blind cave fish (*A. jordani*) (*42*), which is adapted to cave dwelling. Our experiment showed that the ganglion cells of this fish were also capable of dark (excision) repair.

Using a fish brain system, we compared DNA repair in 5 species of small fish, and UDS was demonstrated in all in response to 2 carcinogens, 4HAQO and MMS. This implies that most fish have the ability to repair both UV-type damage and alkylation of DNA.

We conclude from this work that most species of fish have genetic information for excision repair, but this information may not be fully expressed in some somatic cells. On the basis of these findings, we suggest that further studies on DNA repair after treatment with various carcinogens to a wide range of species of fish should be helpful in detecting the most suitable species for use in screening tests for environmental carcinogens.

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The Amazon Molly, *Poecilia formosa,* as a Test Animal in Carcinogenicity Studies: Chronic Exposures to Physical Agents[1, 2]

Avril D. Woodhead, Richard B. Setlow, *and* Virginia Pond[3]

ABSTRACT—The Amazon molly, *Poecilia formosa,* a small, live-bearing fish native to Texas, has an unusual mode of reproduction that makes it highly suitable for carcinogenicity studies. The species is easy to rear and breed, and withstands handling well. Large broods of young are born, numbering up to 90 when the parent fish are fully grown. The Amazon molly reproduces gynogenetically; eggs are activated after mating with males of closely related species. The male makes no genetic contribution; sperm provides only the stimulus for egg development. The offspring of a female comprise a clone with genotypes identical to that of the mother's so that cell and tissue transplants made between members of the clone are not rejected. We used the Amazon molly as an animal model to show that damage to DNA caused by UV and ionizing radiation and by certain chemicals results in tumor development. Thyroid cells were taken from donor mollies, treated precisely in vitro with the agents, and then injected into homologous recipients. Eight months later, recipient fish had developed large thyroid tumors. The genetic homogeneity of the clone also makes the Amazon molly invaluable in acute or chronic bioassays because variability in the responses of the individual fishes is minimal. Therefore, a limited number will suffice to give statistically significant results. The findings from a study of chronic exposure to asbestos are also described. — Natl Cancer Inst Monogr 65: 45-52, 1984.

Almost one-half a century ago, Carl and Laura Hubbs announced their discovery of a small viviparous cyprinodont fish, apparently reproducing gynogenetically (*1*). This event greatly excited the imagination of the zoological world for, at that time, parthenogenesis had been encountered only among the invertebrates. They had found the fish *Poecilia formosa,* the Amazon molly, living in streams and rivers in northeastern Mexico and the southern tip of Texas. Among thousands examined not a single male was seen. For a quarter of a century, the Amazon molly stood alone as the only naturally occurring unisexual vertebrate until the mid-1950s, when the existence of several others was claimed among fishes, salamanders, and reptiles (*2*).

GENERAL BIOLOGY

Species Evolution

In a series of experimental breedings made over the next 12 years, Hubbs and Hubbs (*3*), using about 8,000 fish, established that the Amazon molly reproduces gynogenetically, i.e., by parthenogenetic development initiated by sperm. Females do not become pregnant unless they are mated to males of the same or closely related genera and sperm is transferred, stimulating the development of the ova. The male makes no genetic contribution to the offspring; whichever species of male is used for mating, the young are genetically identical to the female parent, i.e., inheritance is matroclinous. In the wild, the Amazon molly usually mates with males from the genus *Poecilia,* but it will also bear young after mating with males from the genera *Gambusia, Allopoecilia, Limia,* and *Lebistes,* although the mollies are about ten times more fertile when mated with poeciliid males than with the others. No young result from pairings with males of *Quitana, Platypoecilous, Xiphophorus, Pseudoxiphophorus, Heterandria, Glaridichthys, Poecilistes,* and *Micropoecilia* (*3*). Hubbs (*4*), Haskins et al. (*5*), and Abramoff and associates (*6*) demonstrated that the Amazon molly is a hybrid species originating from a parental cross between *Poecilia latipinna* and *P. sphenops.* In nature, the species exists as several distinct clones often with 80% of the population belonging to one large clone and the remaining 20% to several smaller ones. In each geographical location, the Amazon molly is intermediate between the local forms of the parental species in features such as the depth of the body, the position and size of the dorsal fin, and the number of fin rays. Kallman (*2, 7*) and Darnell et al. (*8*) have shown that histocompatibility is complete among the descendants of a single original female. The clonal structure of a population in any particular area remains constant for periods up to 9 years (*2*).

It has been reported that occasionally a male molly may occur in the wild (*5*). However, Kallman (*9*) has suggested that these fish are actually genotypic females and that their male phenotype results from abnormalities during their development. Haskins and his colleagues (*5*) demonstrated that male secondary sexual characteristics, such as elongation of the pelvic fin, could be induced in adult Amazons by treatment with testosterone. Hamaguchi and Egami (*10*) showed that this modification did not involve sex reversal of the gonad.

Normal sexual reproduction also may take place from time to time. A few hybrid fish, which could be singled out

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ABBREVIATIONS: PRL = photoreactivating light; J = joule.

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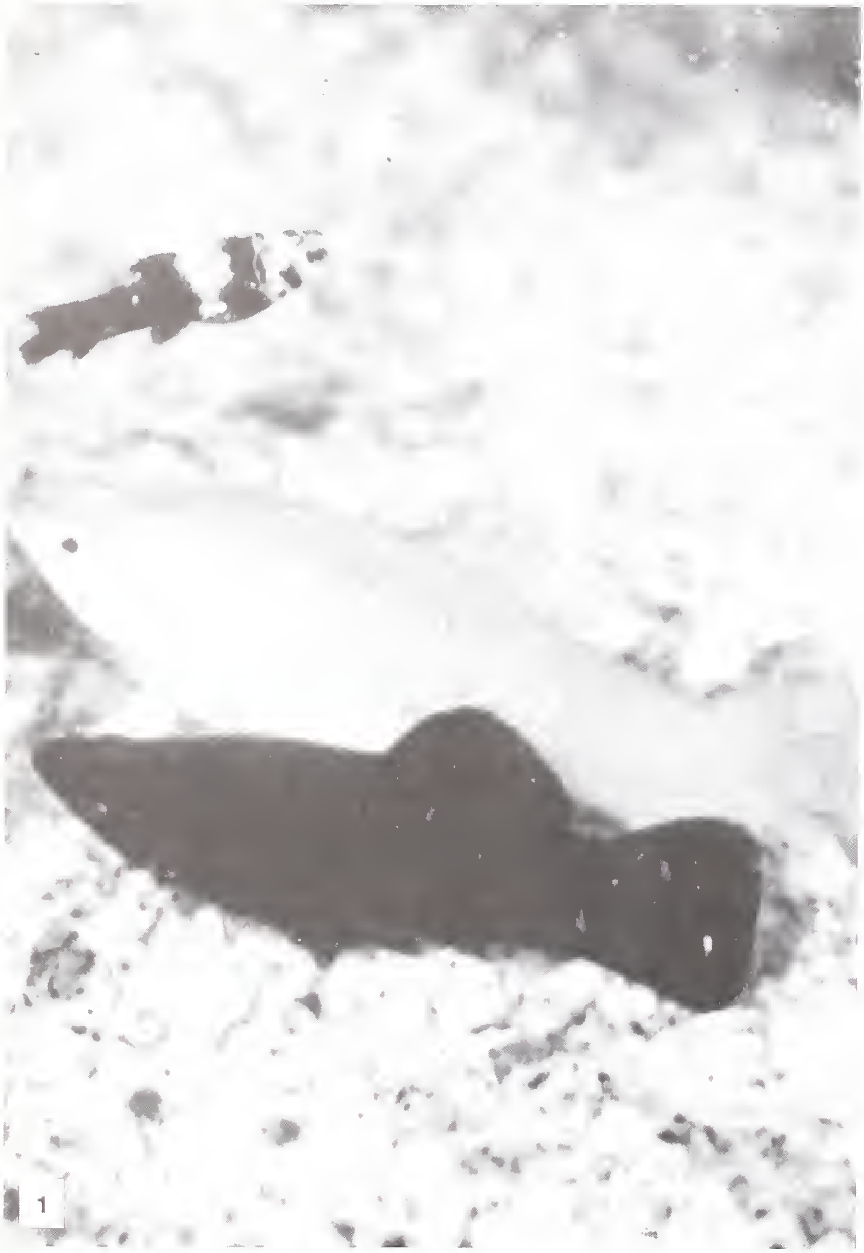

FIGURE 1.—The Amazon molly, the black molly (♂), and the hybrid offspring.

by their color pattern, have been born in our laboratory population from crosses between the male black molly *Gambusia yucatana* and the Amazon molly (*11*). Hybrids are rare, however, and generally seen only among the broods of large fishes when the number of young born is 70 or more (fig. 1).

Genetics

The Amazon molly is a permanent diploid, having a chromosome number of 46, identical with its congeneric species *P. latipinna* and *P. sphenops*. The nuclei of all 3 species contain similar amounts of DNA, about 1.6×10^{-12} pg (*12*). Kallman (*2*) favored the view that the offspring develop from diploid eggs that fail to undergo reduction division. Later, Schultz and Kallman (*13*) suggested that, as an alternative mechanism, the premeiotic chromosomes double without a nuclear division, so that the primary oocytes are tetraploid and become diploid after undergoing meiosis.

Triploid hybrids between *P. formosa* and its sympatric species *P. sphenops, P. vittata,* and *P. mexicana* have also been reported (*12–14*). The triploid fish are all females, are fertile, and transmit the triploid genome to their all-female offspring. In nature, clones of triploid mollies arise sporadically, and seasonal fluctuation is marked in the number of such clones, which may range from 0 to 100% of the local population at any site. Rasch and Balsano (*12, 15*) believe that stressful environmental conditions, such as high temperature, may cause the production of triploid fish.

HUSBANDRY

Life Cycle

The Amazon molly is a naturally occurring species, living in a wide range of environments and competing successfully with its congeneric bisexual species. It flourishes at a range of salinities from fresh to brackish water and at temperatures from 16° to 26° C. The Amazon molly shows none of the "depression" often associated with inbred species (such as lowered vitality and depressed reproductive performance), is disease-resistant, and withstands surgical procedures well. It is short-lived, yet usefully has a well-defined, postreproductive period.

Young may be born at any month of the year, but the number of pregnant females usually declines during the late fall and winter, and the interval between successive broods becomes longer. The largest percentage of young in our laboratory population is born during the months of May to September (fig. 2). With manipulation of the seasonal day/night cycles during the winter (from October through February) to one of 16 hours/8 hours throughout, we have obtained broods up through December, although the numbers of young born in January and February does decrease. The extended light period primarily appears to prevent the lengthening of the interval between broods which normally occurs in the winter months.

Small primary oocytes are present in the ovary immediately after a brood has been born. They grow rapidly in size and are filled with yolk ready for stimulation in about a month. Gestation lasts approximately 1 month. At birth, the fish measure approximately 10 mm and weigh 0.2 g. Their subsequent rate of growth depends on the rate of feeding and the space available to them. Under our normal aquarium conditions, growth is nearly isometric for the first 2 to 3 months of life; the maximum length achieved is 65 mm and maximum age is 40 months. We frequently keep fish up to the age of 30 months, at which time they are in excellent physical and reproductive condition and show no signs of senescence. After this time, breeding performance gradually declines. Histologically, the first indications are seen when some of the large yolky eggs in the ovary start to show premature fusion of the yolk globules and degeneration of the nuclear membrane. By 36 months, all the large oocytes are abnormal in some way and many are in the process of resorption. Shortly thereafter, atretic changes become established in the previtellogenic eggs, and gradually all the oocytes disappear from the ovary (*16*). A brief period of reproductive senescence occurs before the end of the life-span. We see few consistent outward signs of senility until a few weeks before death, when the fish rapidly start to lose weight and become sluggish. Also, fish older than 36 months generally can be recognized by the appearance of melanin spots on the peduncle of the tail.

Fecundity

In common with most teleost fishes, the number of young increases as the length and weight of the female increases. About 20 offspring are born at the first gestation, and brood size increases to about 80 when the fish are fully grown (fig. 3). We find no seasonal differences in the size of the broods, in the size of the individual young, nor in their subsequent rate of growth.

Rearing Requirements

We have been rearing and breeding the Amazon molly on a large scale in the aquarium facility of the Biology Department for the past 5 years. At present, we have 110 fish tanks each with a capacity of 30 gallons for holding the experimental fish; generally we keep 20 fish in each, at a density of about 1 fish per gallon. At any one time we can

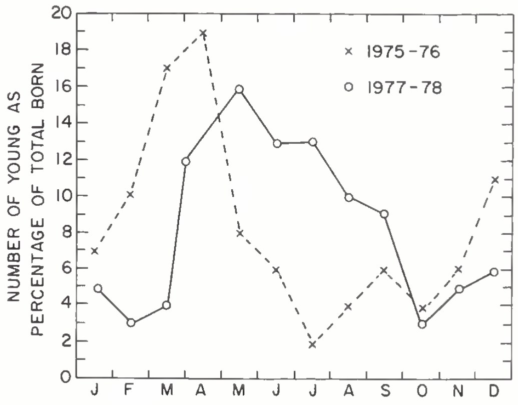

FIGURE 2.—The numbers of young born monthly; data for 2 yr. Figure is reproduced from (*11*).

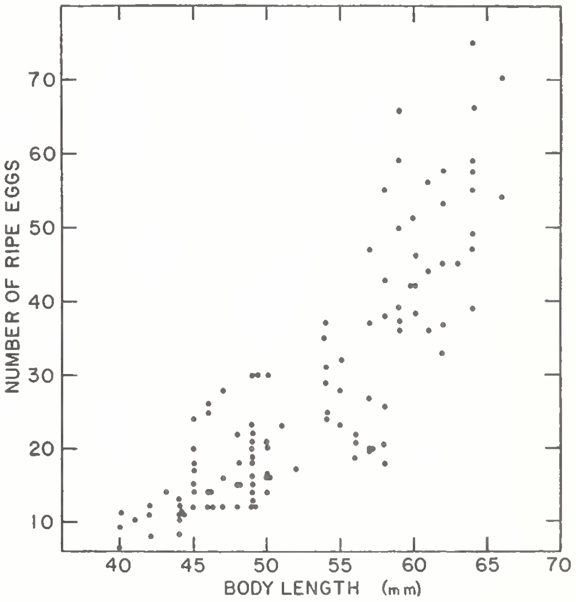

FIGURE 3.—The fecundity-length relationship in the Amazon molly.

hold about 2,000 experimental fish. Water temperature is maintained at 25° ± 2° C and salinity at 2 parts per thousand, which is obtained by adding 1 g sea salt/liter of water. This addition of sea salt also provides an iodine-rich environment for the fish and prevents the development of goiter. Aeration of the water is vigorous.

The aquarium system is semiautomated. Water is pumped from a deep well into an overhead water tank, where it equilibrates with the temperature of the aquarium room. The aquaria on each side of the room are connected to a single circulating system, and thus we can control the total environment of the fish throughout the system. Water runs in and out of the fish tanks, passing into and through a separate large gravel and sand filter bed, through a UV sterilizer, and back into the aquaria. Water is slowly run to waste, so that buildup of nitrogenous wastes in the tanks is avoided; in this manner, the original water is entirely replaced by the end of 40 days. Debris is removed daily from the bottom of the tanks by suction tube. Adjacent to the main aquarium room is a smaller room in which we currently maintain about 90 breeding fish in 30 tanks, keeping 3 Amazon mollies and 1 male black molly in each of the 10-gallon tanks.

Disease

Because water quality is carefully controlled, the fish remain comparatively free from disease. Premature mortality rate is low; over 95% of the untreated fish survive until the end of our studies, and we have few unexpected deaths in our experimental fish. However, inasmuch as a few early deaths do occur, it was important at the beginning of this work that we have a knowledge of their cause. Therefore, Hart et al. (*11*) analyzed by gross pathology or histopathology the cause of early death of fish over a 2-year period in about 100 of the tanks. By far the most frequent cause of death (75%) in nonexperimental fish was by infection with *Mycobacterium fortuitum*. The lesions are characteristically large reddish-blue granulomas that stained positively for *Mycobacterium* spp. Approximately 15% died from a fungal infection observed as a white fluffy growth, principally on the caudal fin. The remaining 10% die from a wide spectrum of causes, ranging from an occasional protozoan infection to various bacterial infections. In only 2% of the deaths was there no apparent cause, and in only 1 instance out of several thousand was a cancer confirmed, which was diagnosed as a melanoma.

No treatment appears to effect a cure for *Mycobacterium* infections. Isoniazid at a concentration of 0.7 mg/ml acts as a prophylactic and inhibits the growth of lesions but will not reverse the disease. At a concentration of 20 mg/gallon, Maracyn introduced into the water daily for 4 days seems to eliminate the fungal infection. An added advantage of this treatment seems to be its wide-range effect on several bacterial infections and its ability to prevent other infections (*11*).

Diet

Since 1975 when the population of Amazon mollies was first established at Oak Ridge National Laboratory, the fish have been fed from birth on the same standard diet. The food is especially made up for us by Harry's Aquarium, Oak Ridge, Tennessee, and with large quantities made up at any one time, we are assured of quality control. The food is high in protein and has ample amounts of essential vitamins and minerals. Iodized salt is added to prevent the development of goiter. Measured amounts of food are given daily, and the food is consumed within 10 minutes of feeding. Hart et al. (*11*) initially used commercial fish flakes but changed to this diet because of the high cost of the flakes and a slower growth rate of the fish.

ADVANTAGES OF THE AMAZON MOLLY AS AN ANIMAL MODEL

Molecular Studies

The value of a fish as a test organism is greatly enhanced if it has a particular unparalleled advantage. The unique feature of the Amazon molly is that it exists in nature as a clone and so is invaluable for studies of the molecular mechanisms of carcinogenesis. For this reason, it was chosen by Setlow and Hart for their studies of the in vivo effects of biological damage to DNA by physical and chemical carcinogens, particularly by UV radiation.

The evidence is excellent that most human skin cancer arises from solar UV radiation, and the inference that DNA is the target molecule is good (*17*). Many kinds of photoproducts are made in cells by UV radiation. One of these products, cyclobutylpyrimidine dimers (usually termed pyrimidine dimers), has been shown to have mutagenic or lethal effects, or both, in microorganisms, by virtue of the fact that these effects do not occur if the dimers are monomerized by a photoreactivating enzyme in the presence of light of a greater than 320-nm wavelength (*18*). Using the imaginative experimental stratagem outlined below, Hart and Setlow (*19*) showed that this particular molecular lesion can give rise to tumors in the Amazon molly. Because only pyrimidine dimers and no other photoproducts are enzymatically photoreactivated, they could thus change the relative proportions of pyrimidine dimers by modifying the radiation dose with and without photoreactivation and observe how tumor induction was affected.

Donor fish, aged 2 to 3 months, were killed on ice, and washed thoroughly in 2.6% sodium hypochlorite, cold 95% ethanol, and then sterile fish Ringer's solution. Scales were removed from the lower jaw, and the thyroid gland was dissected. In the Amazon molly, as in most teleost fishes, the thyroid gland is a diffuse organ, made up of numerous single follicles scattered in the loose connective tissue around the ventral aorta and the bases of the gill arches. Individual follicles are regular and rounded, with squamous or cuboidal follicular cells, surrounding a homogeneous colloid. Thyroid follicles made up about 50% of the tissue removed, which included muscle and cardiac tissue from the urohyal and heart, respectively. The tissue was homogenized in fish Ringer's solution to give a single-cell suspension, and aliquots were then exposed to different doses of UV radiation or to UV radiation plus PRL. Treatment of cells in vitro allowed precision in the exposures. Twenty-microliter samples containing 2.5×10^5 cells were then injected into the abdominal cavities of isogenic recipient fish, aged 3 to 4 months.[4] The fish were killed about 8 months later, serially sectioned, and the lesions scored. The fraction of fish with thyroid growths depended on the UV dose (fig. 4). Profuse thyroid growth was seen in most of the fish given a single injection of thyroid cells that had been exposed to UV radiation at an average dose of 10–20 J/m^2 (254 nm). The growth consisted of a solid mass of thyroid tissue lying in the midline of the floor of the mouth, partially enclosing the anterior branchial arches. In fish with well-advanced growths, thyroid tissue had spread to enclose the first two gill filaments, leaving only the tips of the gill lamellae free; it had also encroached upon the posterior gill arches. Microscopically, the growth consisted of numerous closely packed follicles, similar in appearance to those in the normal thyroid gland. The thyroid growth was highly invasive, and both muscle and bone were eroded and destroyed. The histological findings were consistent with the view that a tumor had been induced by the injection of UV-treated cells.

When PRL was given 5 minutes after the UV treatment, a large decrease in the number of fish with growths resulted, whereas, when it was given before UV exposure, no effect on the incidence of growth was noted (table 1). These data indicate that pyrimidine dimers in DNA give rise to tumors (*21*). By relating the incidence of tumor formation to UV exposure, we calculated the probability that a random dimer will transform a cell. Transplantation experiments, in which cells taken from a well-developed thyroid tumor were injected into fish of another clone and there initiated the growth of a similar tumor, bore out our contention that cell transformation had occurred (*21*).

Damage to DNA caused by ionizing radiation and by chemicals such as *N*-acetoxy-2-fluorenylacetamide also resulted in tumor development and hematopoietic lesions in the Amazon molly (*19, 22*). We consider that data obtained using the Amazon molly as a test organism to be verifiable and realistic; meaningful extrapolation of the data has been made (*21*). We recently explored the possibilities of using fish cells and tissues in in vitro studies of DNA repair mechanisms. Fish cells compare favorably with cultured cells from mice, and, for photoreactivation, have a repair capability closer to that of human cells (table 2).

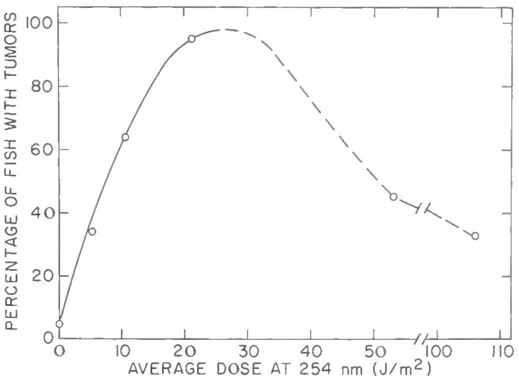

FIGURE 4.—Dose-response curve for the induction of thyroid tumors in the Amazon molly. Figure is reproduced from (*21*).

TABLE 1.—*Fraction of fish with tumors 8 mo after injection with UV-irradiated thyroid cells with or without PRL*[a]

Expt No.	Treatment	Fraction of fish with tumors	
		Gross	Histological
1	UV, 12 J/m^2	34/34	29/29
	2.5 min PRL + UV	26/26	22/22
	5.0 min PRL + UV	48/50	22/23
	UV + 5.0 min PRL	1/42	0/6
2	UV, 24 J/m^2	40/40	10/10
	5.0 min PRL + UV	38/40	10/10
	UV + 5.0 min PRL	0/22	0/10
	Untreated	0/22	0/10

[a] Data were adapted from Hart et al. (*21*).

Bioassays

In addition to its exceptional value in molecular studies and for tissue and cell transfer studies, the clonal nature of the Amazon molly confers special benefits in bioassay studies. The genetic homogeneity of the clone ensures that the variability in the physiological, metabolic, and behavioral responses of the fish to environmental change is minimal. Therefore, comparatively few animals are needed in each experiment to give a statistically significant result, and the results are reproducible.

A further advantage in the use of a clone in bioassays relates to the need for background information concerning the normal anatomy and histology of animal test systems. An essential requirement for an animal model is that there should be available a thorough knowledge of its normal physiology and morphology and of the occurrence and incidence of spontaneous tumors and diseases throughout its life-span. The lack of such information for fish and other cold-blooded vertebrates is particularly apparent when the situation is compared with that for mammals and other laboratory animals, for which the literature is detailed. The

TABLE 2.—*Extent of DNA repair in human, mouse, and fish cells after treatment with UV or* N-*methyl*-N-*nitrosourea*[a]

Cells	After UV		After *N*-methyl-*N*-nitrosourea
	Dimer excision	Photoreactivation	DNA adduct removal
Human	+++	+++ (skin)	+++
Mouse	+	± (skin, neonates)	+
Fish	+	+++	+

[a] +++ = pronounced; + = some; ± = occasional.

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[4] Experiments made with radioactively labeled thyroid cells have shown that 30 min after ip injection, the cells are dispersed in the connective tissue around the aorta. By 1 to 4 hr later, large numbers of cells have accumulated in the thyroid region and a few are still found there 200 hr after the single injection (*20*).

accumulation of such information is a formidable task and, undoubtedly, there are advantages in the use of a homogeneous strain in which the range of inherent variation is restricted. This is especially applicable in low-level chronic studies, when tissue damage may be slight during the early period of exposure and is readily masked among the biological diversity of a heterogeneous population. We have made histological observations on the changes in several organs and tissues of the Amazon molly throughout its life-span and are continuing this survey in concert with our experimental studies.

Chronic Exposures

We have been using the Amazon molly to study the effects of long-term exposures because it is hardy and fares well in long-term studies. In this section, we present our findings from initial histological surveys of our first assay with asbestos and discuss some of the factors which concern us in the interpretation of our results.

Amazon mollies were kept for several months in a suspension of 1 or 10 ppm of chrysolite asbestos (No. 7R FO_2; Johns-Manville, Denver, Colo.). Most fibers in the suspension were between 0.2 and 2.0 μm in length (*23*). The experiments were started when the fish were about 2 months old and measured 20–30 mm in length. Each group of 6 fish was kept in a 10-gallon tank. The animal density was less than our usual carrying capacity, but this modification was deemed essential as the system was a closed one and the water could not be circulated. We were concerned with the possible problems that might arise from deteriorating water quality if a larger fish population was maintained.

The accumulation of toxic metabolites, particularly ammonia and to a lesser extent nitrites, may interfere with the ability of hemoglobin to transport oxygen (*24*). The most reliable method with which one can deal with this problem is to remove the toxic metabolites by continuous gradual renewal of the water. However, this practice can be prohibitive because of the high cost of chemicals, especially in chronic exposures. Therefore, we kept the level of ammonia low by carefully removing debris from the bottom of the aquarium twice a day. The water was vigorously aerated and thoroughly mixed by means of a small aeration pump. The tanks were changed and the asbestos renewed every other month; food was given sparingly, so that none remained uneaten after 3 minutes. Fish were removed from the experiment after exposures of 4 or 6 months, killed in ice water, routinely fixed in "Perfix" (Fisher Scientific Co. Pittsburgh, Pa.), and embedded. Serial sagittal sections were made at 6 μm, stained with hematoxylin and eosin, and the tissues examined for lesions.

Studies with mammals showed that the gastrointestinal tract is the major site of exposure to many environmental contaminants contained in the food and water. Particulates, such as carbon particles, penetrate the intestinal wall into the body principally by way of the epithelium covering specialized areas (known as Peyer's patches) where they are engulfed by macrophages. Few particles enter into the systemic circulation, but, after a period of exposure of about 6 months, particles can be seen in the lungs and in the liver (*25*).

In view of the effectiveness of the mammalian intestine as a barrier to particle penetration, we anticipated that the same situation would occur in fish. Few systemic lesions might develop and even a thorough histological examination might fail to detect them. Therefore, we treated a group of fish with particulate carbon (particle diameter, 20–25 nm) to provide a picture of the distribution of small particles.

Amazon mollies were exposed for periods from 12 to 120 hours to a 0.05% suspension of carbon, and the subsequent pattern of distribution was followed for 12 days. We also injected 0.01 ml of a 10% carbon suspension directly into the peritoneal cavity in a separate experiment. Both treatments showed that carbon was taken up by macrophages mainly at 3 sites: in the head kidney, the heart, and the mesentery. Mesenteric fibroblasts also engulfed and accumulated carbon particles. Large accumulations were seen especially in the intertubular areas of the kidney (fig. 5); we saw no evidence of the subsequent loss of the carbon particles from any site within the 12-day period of observation (*26*).

The pattern of distribution of particulate carbon in the body gave us a base line as to where to look for lesions caused by asbestos. They invariably occurred in the head kidney. Examination of histological sections through the head kidney showed the presence of numerous abnormally large holes, giving parts of the organ a honeycomb appearance. Under high magnification, most of these "holes" were dilated renal tubules, lined by flattened epithelial cells. Some contained cell debris, and some showed a conspicuous development of fibrous tissue around the dilated tubule. The changes in the kidney bore some resemblance to those alterations found in the alveoli of the lungs of humans and animals exposed to asbestos dust, when the lodgment of smaller fibers of 20 μm and less may eventually lead to dense fibrosis, loss of alveoli, and dilation of the remaining bronchioles.

The gills of exposed fish were also characterized by generalized lamellar hypertrophy, with areas of proliferation at the bases of the secondary lamellae. Some fish had telangiectatic secondary lamellae on the gills, lesions caused by the rupture of the retaining pilaster cells joining the dorsal surface of the secondary lamellae to the ventral surface with subsequent infiltration of blood.

Control groups are essential for closed-system studies, for even though the chemical and physical changes in the water are frequently monitored and the water renewed, the observed pathological changes might be associated with other unknown factors. Thus both dilation of the renal ducts and lamellar telangiectasis of the gills may sometimes be seen in fish living in intensive culture or in association with high levels of chemical pollution of water. We observed small areas of hypertrophy and hyperplasia of the gills in 5% of control fish but did not find comparable kidney lesions in any of the control animals. However, high levels of calcium and magnesium in the diet may cause pathological changes in renal architecture. Chrysolite asbestos is a hydrated magnesium silicate, so it is unclear whether the renal lesions are primarily the result of local exposure to the metal or due to physical damage from the sharp asbestos fibers.

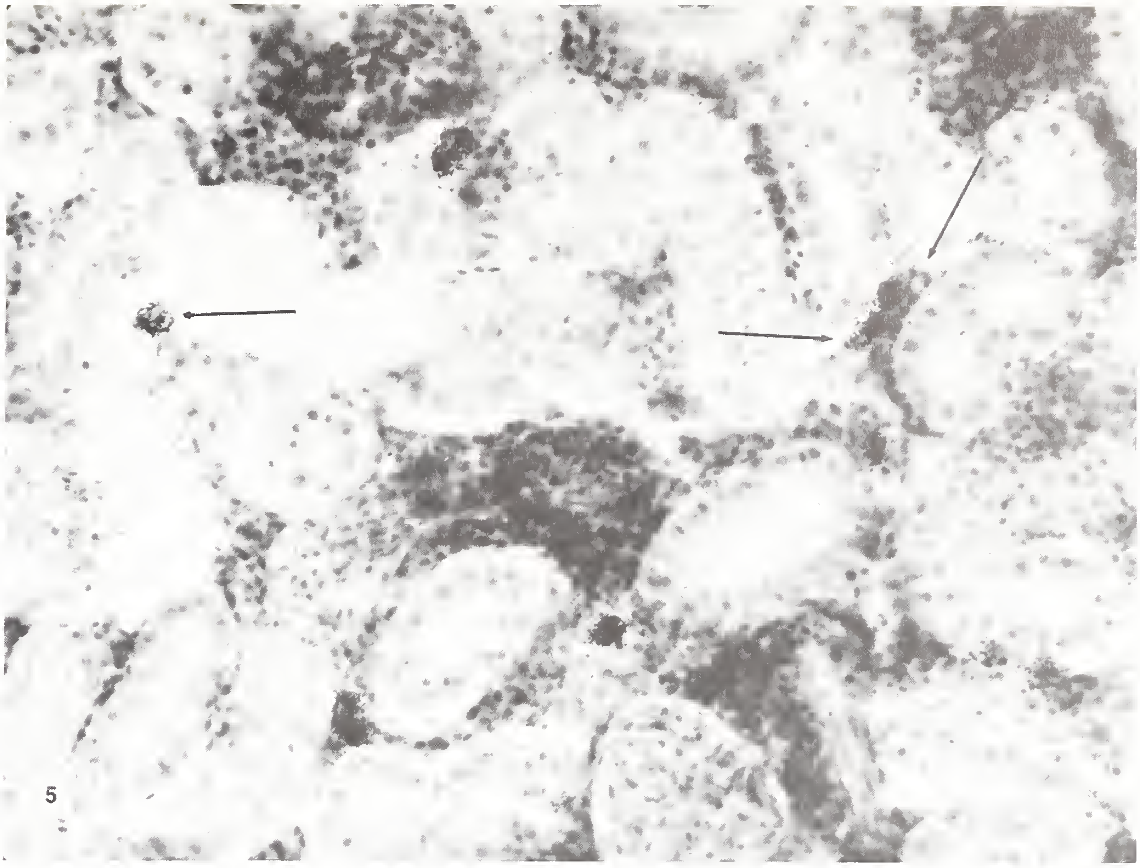

FIGURE 5.—Large aggregations of carbon-containing macrophages (*arrows*) in the intertubular tissue of the head kidney in close proximity to the hematopoietic tissue.

Manpower Requirements and Relative Costs

A rationale for the use of fish in carcinogenicity studies is the significantly lower cost of maintenance and the relative ease of handling and housing large numbers of animals. These economic features are salient when compared with the typical rodent test which requires 3 years and costs more than $100,000 (*27*). Because our system is semiautomated, it can be maintained at full capacity by 1 curator working a half-day schedule for 5 days a week. Over the weekend, the fish are fed but no further maintenance is done. We have calculated the cost of rearing the fish as being $0.28/fish per month, including wages (and fringe benefits) of the curator, feeding, and energy costs. In comparison, the Department of Health and Human Services has several strains of rats and mice available for purchase to researchers, and they calculate that the cost of maintenance of a mouse is $0.60 per month, more than double the cost of the molly. A Sprague-Dawley rat costs $3.39/month. In addition to the lower maintenance costs, the space requirements for keeping a large population of fish are considerably less than for comparable numbers of mammals.

Pathology is a major part of carcinogenicity testing and represents about 40% of the overall costs of a study. There are no shortcuts to a thorough examination of the tissues. For example, a simple survey for metastatic foci from a primary liver tumor in randomly selected lung lobes of mice showed an average incidence of 0 and 2% for females and males, respectively, but sections of their whole lungs at 2 levels revealed over 20% of the mice had metastatic foci (*28*). Pathological examination is generally far less time-consuming and can be much more thorough with small fish, with which serial sections can be made through the entire animal in 4 hours.

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Attainment of Minimal Biological Variability and Measurements of Genotoxicity: Production of Homozygous Diploid Zebra Fish[1, 2]

George Streisinger[3, 4]

ABSTRACT—Zebra fish (*Brachydanio rerio*) are particularly suited for carcinogenicity testing and for the establishment of quantitative dose-response relationships. They are small, prolific, have short generation times, and produce eggs which develop synchronously and can be treated at identical developmental stages. Clones of homozygotes with minimal genetic variability provide a reproducible biological test system. Rapid measurements of the frequency of induced somatic mosaicism (demonstrated with gamma radiation, ethyl methanesulfonate, and ethylnitrosourea) and induced germ-line recessive-lethal mutation provide indexes of exposure. — Natl Cancer Inst Monogr 65: 53–58, 1984.

Laboratory experiments serve not only to identify carcinogens but also to indicate the degree of risk that they pose. To extrapolate from high doses used in the laboratory to lower ones that may be encountered in the environment, one must establish the nature of the dose–response relationship for any given potential carcinogen. The identification of agents which cause cancers as a first-order function of dose is particularly important: Such agents may result in an appreciable number of affected individuals if a sufficiently large population is exposed to even low levels. For this reason, test systems which provide for large-scale animal exposures may prove especially valuable.

The small freshwater tropical zebra fish (*Brachydanio rerio*) is exceptionally well suited for carcinogenicity testing, the establishment of quantitative dose–response relationships, and for the comparison of carcinogenic and genotoxic effects of environmental agents: 1) Dose–response studies and other experiments requiring large numbers of individuals can be performed readily because the fish are small (~3 cm), and several hundred eggs are produced at frequent intervals by each female (*1*). 2) Two- and three-generation studies are facilitated by the 3- to 4-month generation time. 3) Large numbers of eggs at identical developmental stages can be exposed to test compounds for controlled periods because eggs can be fertilized synchronously in vitro and develop outside the mother.

Inherent genetic variability in many populations reduces the reproducibility of tests. To diminish genetic variability, we produced clones of homozygous zebra fish (*2*). The production of homozygotes also provides for a simple assay of genotoxicity.

MATERIALS AND METHODS

Standard type fish were obtained commercially and were thereafter maintained in a closed colony. The *gol-1* mutant was found in our standard-type population. Two- to 3-day old *gol-1/gol-1* fish appear pigmentless, whereas *gol-1+/gol-1+* or *gol-1+/gol-1* fish are covered with large melanocytes. The preparation of homozygous clones is described elsewhere (*2*).

Fish were kept at about 27° C in a 14-hour light and 10-hour dark cycle, at about 1 fish/liter in tap water, which was filtered through charcoal filters and supplemented with 1 g sea salts (Instant Ocean, Aquarium Systems, Mentor, Ohio). (With recent batches of Instant Ocean sea salts, the concentrations described here proved toxic. At present, we supplement with 63 mg sea salts/liter.) Standard-type fish were maintained at a ratio of 1 female to 2 males, whereas homozygous fish were maintained with an equal proportion of males because higher ratios resulted in stress (with poor appearance of females, a higher proportion of eggs develop abnormally). For the purpose of egg collection, females were segregated from males about 17 hours before the beginning of a light cycle. Shortly before fertilization was due, males were anesthesized in 0.2 mg ethyl *m*-aminobenzoate methanesulfonate/ml (Finquel; Ayerst Laboratories, New York, N.Y.) adjusted to pH 7.0 with Tris(hydroxymethyl)aminomethane. Sperm were expelled by gentle pressure applied to the abdomen with smooth forceps and collected with a capillary pipette. Sperm from 4 or 5 males were pooled in 0.4 ml of cold Hanks' saline to give a concentration of about 10^8 sperm/ml. Sperm in a watch glass were exposed to UV light from a 43-cm germicidal tube (Sylvania Corp., Millburn, N.J.) at a distance of 29 cm for 2 minutes. During the first 90 minutes

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ABBREVIATIONS: EMS = ethyl methanesulfonate; ENU = ethylnitrosourea.

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after the beginning of the light cycle, eggs were expelled from anesthesized females in 3.5-cm plastic petri plates (Lux Scientific Corp., Newbury Park, Calif.; some batches of another brand proved toxic to zebra fish embryos) by gentle finger pressure. Eggs were activated by the addition of 0.05 ml of sperm suspension and 0.5 ml of water [glass-distilled and supplemented with 1 g sea salts/liter (Instant Ocean)] at 28.5° C, followed by further water after about 30 seconds. Activated eggs were incubated at about 28.5° C.

A few hours after activation, infertile or dead eggs were removed, and groups of about 50 eggs were transferred to 250-ml beakers containing 100 ml water. Developing embryos were examined daily and dead embryos were removed. At about 9 days postfertilization, free-swimming larvae were transferred to mouse cages and at about 2 weeks they were transferred to aquaria.

Fish 1 month old and more were fed twice a day with a rotation of: Tetra growth food (Tetra), Oregon test diet (*3*), Oregon moist pellets (Bioproducts Inc., Warrenton, Ore.), liver-spinach pâté (*4*), and brine shrimp (*Artemia*) nauplii (eggs from Aquarium Products, Glen Burnie, Md.). If adult brine shrimp are available, they can be substituted for the Oregon test diet, moist pellets, and brine shrimp nauplii. Fish aged 4–14 days were fed paramecia, and 9- to 30-day-old fish were fed brine shrimp nauplii.

RESULTS

Genetically Uniform Fish

Simple procedures have been developed for the large-scale production of homozygous fish. Eggs are activated with sperm made genetically impotent by exposure to UV light. The maternal haploid complement of chromosomes is permitted to duplicate once, and partitioning of the duplicated chromosomes into 2 cells during mitosis is prevented by hydrostatic pressure or heat shock treatment (*2*). These treatments result in a diploid cell with 2 identical sets of chromosomes, both derived from the mother. The subsequent cycles of chromosome replication and cell division proceed normally and give rise to diploid fish with 2 identical (homozygous) sets of chromosomes in each cell.

In treated eggs of our initial fish population only a few percent developed into adults. Some losses were due to the treatment itself, others to the expression of deleterious genes in the homozygotes. To produce hardier homozygous fish, we mated homozygotes derived from the initial population (and therefore with different genetic constitutions) to obtain hybrids with a potentially improved gene pool. From these hybrids, a new generation of homozygous fish was produced (by pressure treatment), and the selective breeding protocol was continued for several generations (*2*). Some homozygous fish in each generation were males and females, which facilitated matings that produced selected hybrid populations. More than 25% of heat-shocked or pressure-treated eggs from fish of the third generation of selected hybrid populations developed into fertile adults.

Appropriate genetic tests (*2*) demonstrated that the treatments indeed produced homozygotes: No significant heterozygosity was contributed by the UV sperm ($<10^{-3}$) nor by nondisjunction of maternal chromosomes ($<10^{-2}$).

Clones of homozygotes were produced from individual homozygous females by the heat shock or pressure treatments (*2*). The chromosome sets in all these fish are identical because they are all derived by replication from a single haploid chromosome set. In the clones we use at present (C29 and C32), most fish are females; males are produced by exposure to 1.7×10^{-7} M 17α-methyltestosterone (Sigma Chemical Co., St. Louis, Mo.) during the first 2 weeks after fertilization. Clones are propagated by ordinary crosses of males and females.

The clones C29 and C32, though resembling standard populations of fish, are less hardy and more demanding nutritionally. For optimal egg production in homozygotes, a rotation of supplemented foods was required. To produce genetically uniform populations of fish hardier than these homozygotes, we crossed members of these 2 homozygous clones. The resultant progeny were heterozygous but isogenic (genetically identical to one another). One can produce identical populations at any time by crossing the same 2 clones.

Measurements of Genotoxicity as an Index of Exposure

Induction of Mosaicism

To determine whether potential mutagens and carcinogens were reaching target tissues, we developed a rapid test of genotoxicity based on similar tests with mice (*5, 6*). Heterozygotes for a recessive pigment mutation were exposed to mutagens during embryogenesis, and the appearance of patches of mutant tissue was measured later during development, at 2 or 3 days postfertilization.

The test utilizes the recessive golden (*gol-1*) mutation (*2*). Homozygous *gol-1/gol-1* fish are pale at 48 hours after fertilization, in contrast to standard-type (*gol-1+/gol-1+*) or heterozygous (*gol-1/gol-1+*) fish, which are pigmented and exhibit dark pigmented retinas (fig. 1). Mosaicism is scored most readily in the pigmented retina of the eye where even a few golden cells can be detected unambiguously (fig. 2). Treatment of heterozygous *gol-1/gol-1+* blastulae with gamma rays (fig. 3), EMS (Eastman Kodak Co., Rochester, N.Y.; fig. 4), and with ENU (Pfeiffer and Baltz, Stamford, Conn.; fig. 5) produced an appreciable fraction of mosaic individuals. We conclude that these agents effectively reach cells in the blastulae and cause genotoxic effects.

The presence of unpigmented (presumably *gol-1* or *gol-1/gol-1*) cells in mosaics may have arisen by any of three mechanisms: mutation, somatic crossing over, or the loss of the chromosome which bears the *gol-1+* gene. We have not distinguished among these alternatives. Higher than first-order dose responses were observed for gamma rays and EMS, as observed for somatic crossing over with X-rays in *Drosophila* (*7*). The higher than first-order kinetics observed with EMS was not altered by treatment of blastulae at an earlier age, nor by manual puncture or removal of the egg chorion before treatment.

We found no induction of mosaicism with diethylnitrosamine. Therefore, it either fails to penetrate the eggs or is inactive. Furthermore, this nitrosamine in zebra fish

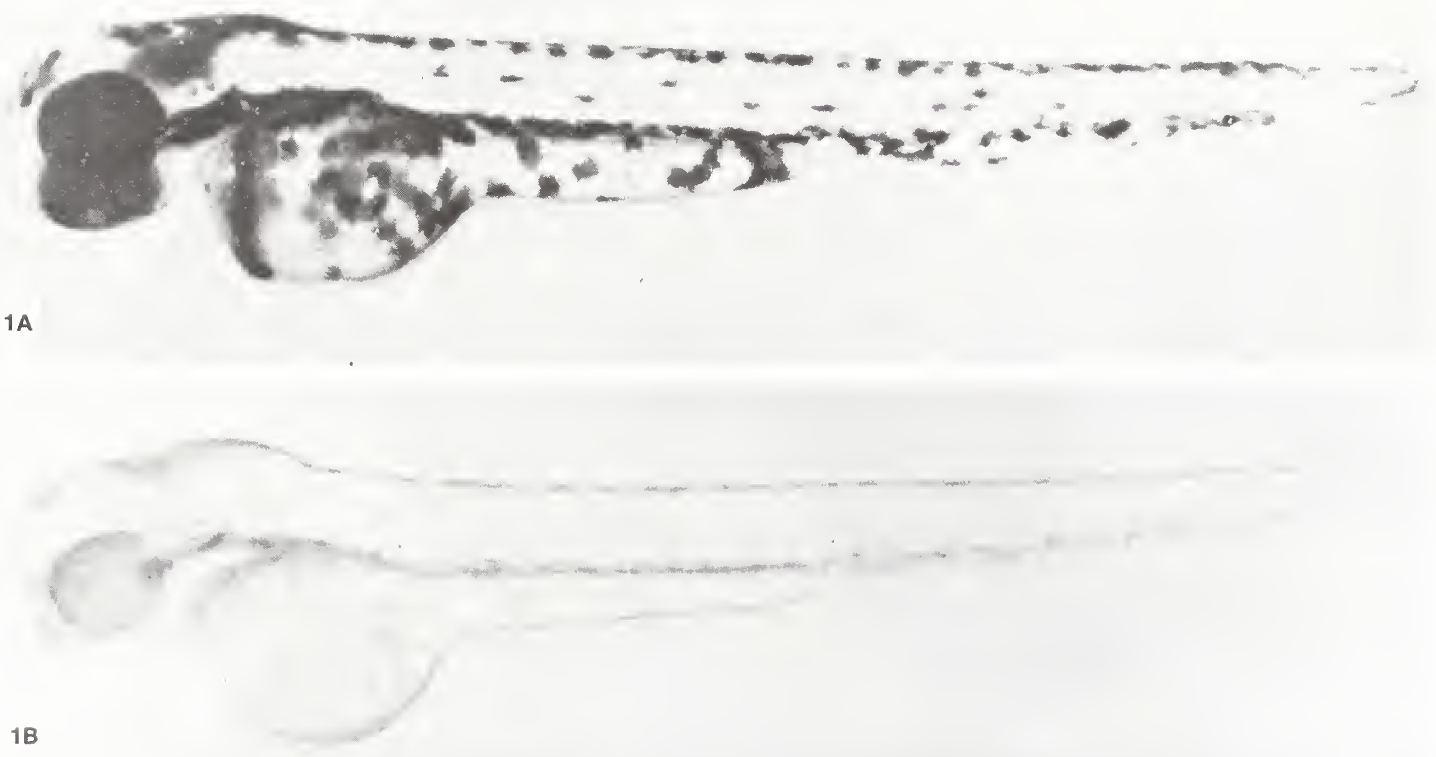

FIGURE 1.—Three-day-old standard-type (A) and golden zebra fish (B) (~3.8 mm long).

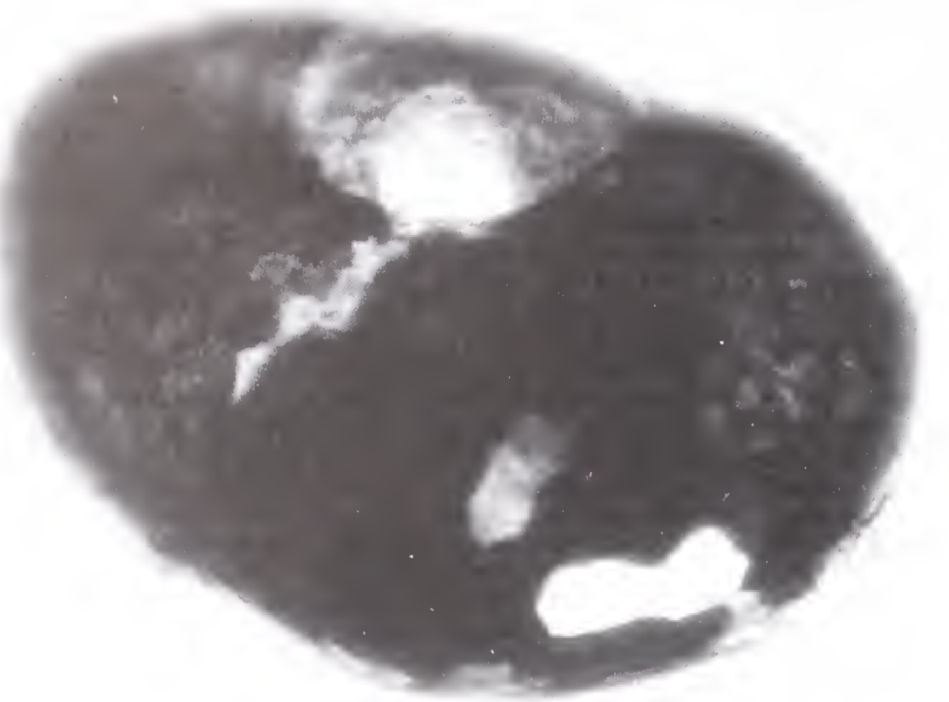

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FIGURE 2.—Mosaic eye.

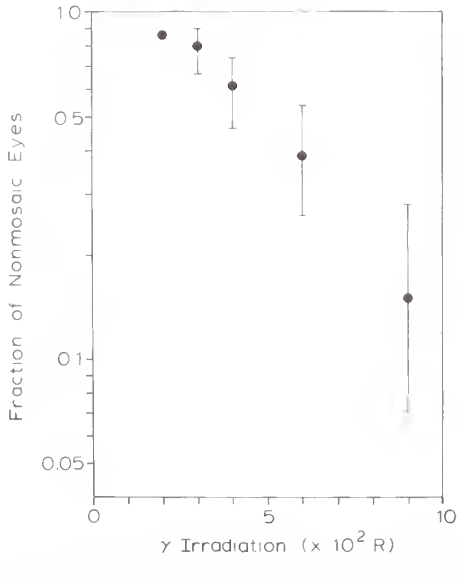

FIGURE 3.—Blastula-stage heterozygous *gol-1/gol+* embryos, incubated at 28.5° C for 300 min after fertilization, were exposed to the indicated doses of gamma rays (at a dose rate of about 1,000 roentgens/min from a cesium-137 source (Model M Gammator, Radiation International, Inc., Parsippany, N.J.). Three days later, the embryos were fixed in 10% formaldehyde in 0.1 *M* phosphate buffer (pH 7.0) and were subsequently inspected with the aid of a low-power dissection microscope. The few eyes which could not be classified unambiguously were dissected and examined under higher power. *Vertical bars* in this and subsequent figures indicate 95% confidence limits. From *left* to *right*, points represent sample sizes of 28, 54, 76, 64, and 52 eyes, respectively.

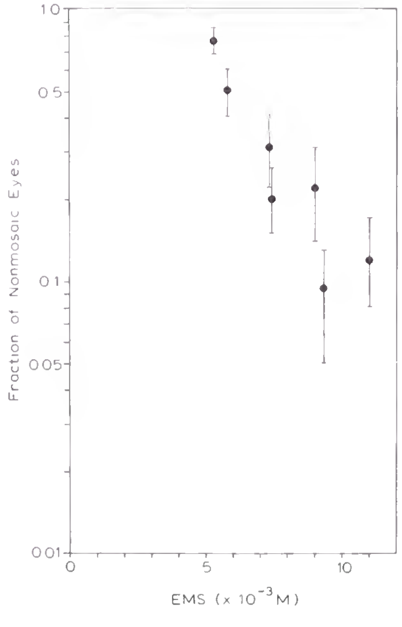

FIGURE 4.—Blastula-stage heterozygous *gol-1/gol+* embryos, incubated at 28.5° C for 300 min after fertilization, were incubated in the indicated doses of EMS for 90 min. The EMS was distilled under vacuum and stored in a desiccator at −20° C until use. Solutions were prepared in water supplemented with 1 g sea salts/liter. From *left* to *right*, points represent sample sizes of 154, 94, 140, 194, 126, 184, and 236 eyes, respectively.

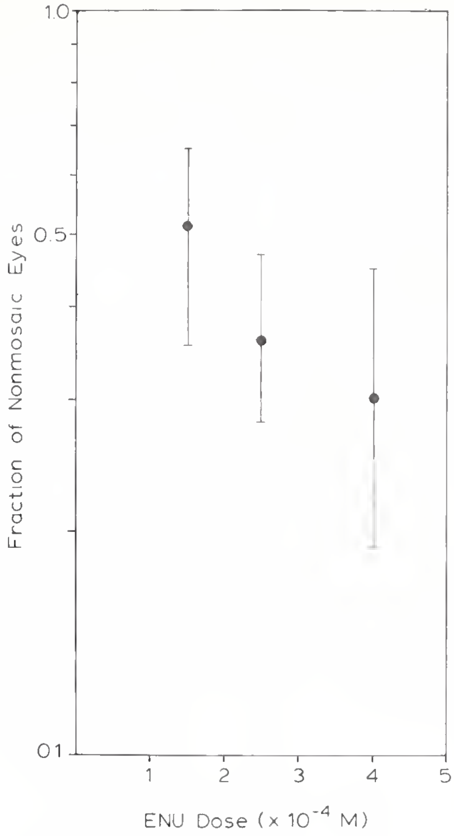

FIGURE 5.—Blastula-stage heterozygous *gol-1/gol+* embryos, incubated at 28.5° C for 160 min after fertilization, were incubated in the indicated doses of ENU for 50 min. Solutions of ENU were prepared in 0.02 *M* phosphate buffer (pH 6.1) and used at a further dilution of at least 1:25. From *left* to *right*, points represent sample sizes of 82, 82, and 12 eyes, respectively.

may be mutagenic only after metabolic activation, and their embryos at the stage of our treatment may lack the ability to metabolize xenobiotic agents.

Induction of Germ-line Mutation

In the determination of whether mutations have been induced in the germ line by treatments that gave rise to mosaicism, treated isogenic embryos were raised to maturity, and the frequency of recessive lethal mutations was measured among their progeny.

The frequency of recessive lethals induced by ENU was measured with eggs of treated and untreated fish made homozygous by activation with UV sperm and exposure to heat shock. Lethal mutations in the germ line of the mother will be homozygous when present in the progeny and will result in their death. Scoring is completed within a week. Most fish that survive for 5 days postfertilization (the time of swim bladder formation) reach maturity. Golin, in our laboratory, found that the fraction of surviving homozygotes was indeed lower among the progeny of ENU-treated fish than among the progeny of the untreated. On the basis of the reduced survival, he calculated that eggs produced by ENU-treated fish carried a mean of 0.4 recessive lethal mutations. The presence of lethal germ-line mutations was confirmed by examination of the homozygous progeny of F_1 individuals produced by the crossing of treated fish with untreated ones. Induced germ-line mutation frequencies can thus be measured readily in zebra fish. Studies of chemical mutagens are being extended to eggs, sperm, and adult males in collaboration with Golin, Walker, and Grunwald.

Germ-line, specific-locus mutations and recessive lethals were induced efficiently by gamma-ray treatments of zebra fish eggs and sperm (*8*) and blastulae (*9*). We expect that zebra fish will prove useful for further studies of mutagenesis and also of carcinogenesis.

DISCUSSION

Genetically uniform zebra fish populations can be expected to serve much as have inbred mouse or rat strains in reducing variability in tests performed at different places or times.

Many clones of homozygous zebra fish contain both males and females. The mechanisms of sex determination in these fish differ from those observed in mammals. The sex ratio in broods of certain fish species has been strongly influenced by the environment (*10*).

The rapid tests of genotoxicity we have described may prove useful in demonstrating that putative carcinogens have reached at least some target tissues. The fact that we do not understand the mechanism by which mosaics arise does not diminish the utility of mosaics as an index of exposure.

The observation that EMS induces mosaics with much higher than first-order kinetics may be partly attributable to the fact that it produces a larger proportion of chromosome breaks and complex alterations than does ENU (*11*).

Treatment of blastula stages will detect only those agents that penetrate the egg easily and do not require metabolic activation. Agents that do not fulfill these requirements could be tested for carcinogenicity by incorporation into the food or by injection.

We have not examined mutagen-treated fish for the frequency of cancers. Measurements of the frequency of cancers in ENU-treated populations would be especially important because they would permit direct comparisons of the frequency with which this agent induces mutations and tumors. Because of the ease with which large numbers of fish can be treated and raised, zebra fish may prove to be useful for measuring dose-response relationships for carcinogens.

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Usefulness of the Guppy, *Poecilia reticulata,* in Carcinogenicity Testing: Special Advantages and Problems[1]

Edmund J. Zimmerer[2]

ABSTRACT—The guppy, *Poecilia reticulata,* native to fresh and brackish waters of northeastern South America and adjacent islands of the Caribbean, has been introduced throughout much of the tropical and subtropical world. It tolerates comparatively wide ranges of temperature, salinity, and crowding. This hardiness plus high fecundity, live birth, fast growth, and early maturation make it an excellent laboratory organism for disciplines ranging from behavioral studies to toxicity testing. In 4 published carcinogenicity studies, 8 of 11 known mammalian carcinogens produced neoplasms in the liver and occasionally at other sites. The guppy's overall popularity, however, has magnified problems concerning stock origin. The natural variability of this species has been increased by artificially selected breeding and by exotic natural selection pressures of foreign environments on introduced populations. The source of many guppy stocks today is ultimately the pet trade, and a good genetic history of such strains is not often available. Differences in stock origin may account for some differential responses of growth and sensitivity to environmental variables noted in the literature. The establishment of carefully maintained inbred strains for cancer research is the best solution to this problem. — Natl Cancer Inst Monogr 65: 59-64, 1984.

Small animals are frequently used in screening tests for environmental toxicants and as models for the physiological and biomedical effects of such compounds. Many such tests involve simple median lethal dosage techniques over a short time that primarily serve only to show toxic effects by a given substance. Long-term or whole-life cycle "bioassay" tests are usually necessary to reveal sublethal effects on growth, reproduction, and overall health of the test organisms. However, the time and costs involved often make such long-term studies impractical.

This same problem of time and costs versus information also applies to the use of animals in carcinogenicity testing, but much of the difficulty can be solved by the selection of proper test organisms. Because the ultimate goal of such research is usually the determination of the carcinogenicity of specific compounds on mammalian (human) systems, it would seem best if we restrict such testing to small mammals. However, even with the smaller laboratory rodents, the time for reproduction, growth, and space for housing become important factors. The use of small submammalian vertebrates, such as aquarium fish, can be a viable alternative. The guppy, *Poecilia reticulata,* with its small size and ease of maintenance is such an animal.

GENERAL BIOLOGY

The guppy, *Poecilia (Lebistes) reticulata,* is a poeciliid fish native to northeastern South America and adjacent islands of the Caribbean. It is ovoviviparous and shows a striking degree of sexual dimorphism; males exhibit a wide range of color patterns. As a result of these characteristics and its ease of maintenance and propagation, the guppy has become one of the best known and most widely used fish species in both the pet industry and in biological research. Its efficacy in mosquito control has resulted in its being introduced over much of the world's tropical and subtropical areas.

The sexes differ outwardly in size, coloration, and fin morphology. Females are generally larger than males and average around 30 mm in total length, whereas the males are about 22 mm. These figures from Seghers (*1*) represent wild populations. Laboratory stock free from predation pressures and raised under better conditions can attain a significantly larger size. Females of wild stock are generally a uniform gray-brown, but males in contrast are colorful. Males also possess a modified anal fin (the gonopodium) used for sperm transfer to the female, as well as proportionally larger caudal and dorsal fins. An in-depth description of the courtship behavior leading to copulation is given by Baerends et al. (*2*). After an insemination, sperm can be kept alive by the female in folds of the ovary. Therefore, 1 insemination can suffice to fertilize the eggs of several broods.

Three size classes of oocytes can be distinguished in the ovary over the gestation period (*3, 4*). Large oocytes are numerous for the first 8–10 days. If fertilization does not occur during this time, they disappear. Middle- and small-sized oocytes are always present. At the next parturition, some middle-sized oocytes develop into large ones, the middle-sized oocytes are in turn replenished from the large number of small oocytes. During the 20 days after fertilization the embryos develop, and after approximately 1 month from the previous birth, the young are born. A highly vascular follicle wall and respiratory portal system in the length of the embryo's ductus cuvieri (*5, 6*) indicate that embryos derived some of their nourishment from the mother to supplement that from the yolk. Intervals between broods are usually between 28–30 days (*3, 7*) but may vary beyond this range. Brood number can range from 1 to over 100.

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[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

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The young, given the proper conditions, can grow and reach maturity in a surprisingly short time. Signs of sexual maturation can be observed in less than 3 weeks, and it is possible for a female to produce young as early as the eighth week. After completion of sexual maturation, females continue to grow, although at a steadily declining rate. In guppy males, as with those of most other poeciliid fishes, growth dramatically slows after sexual maturity is attained (*8–10*). Under ideal conditions, guppies may live over 3 years. However, a practical life-span of 1–2 years is the norm.

The genetics of color and fin polymorphisms has been studied intensively (*11–18*). Normally, males are heterogametic (XY), whereas the females are homogametic (XX). Early work done largely by Winge and co-workers (*16*) has shown most of the color patterns to be Y-linked. However, some patterns were also shown to be X-linked and autosomally linked. Body coloration is mostly limited according to sex; although females may carry genes for color, the expression is limited to the males. A number of color patterns do exist that are not limited in this way. The Flavus (*17*), half-back (*18*), and the gold or yellow patterns (*19*) are examples of patterns that are expressed in both sexes. The number of such patterns described is increasing as fish fanciers breed for the more colorful fish of both sexes.

Winge (*12*) determined the diploid number of chromosomes for this species to be 46, which has since been confirmed by Iriki (*20*), Post (*21*), and Lodi (*22*). Karyotype analysis by Lodi shows the centromeric position to be terminal in all chromosomes; however, identification of any heteromorphic pair as the sex chromosomes genetically demonstrated by Winge was not possible.

REARING REQUIREMENTS

The guppy does best at temperatures between 22° and 26° C (*14, 23*), but temperatures between 20° and 28° C can be tolerated for prolonged periods. Brief fluctuations above or below these extremes can also be tolerated. At low temperatures (below 20° C), fish become sluggish and mortality begins due to cold shock. An absolute minimum for survival of 15° C was reported by Winge (*14*). At high temperatures (above 30° C) fish can appear either agitated or sluggish and often are seen "air gulping" at the surface regardless of aeration. At 32° C, a mortality of over 50% after 1 week can be expected (*24*). The temperature for maximum growth and lethal temperatures can vary as strains derived from contrasting natural conditions show tolerances to different temperatures. Also, the influence of prenatal and early environments on irreversible nongenetic adaptation can affect temperature tolerance. Gibson (*25*) reported that lethal temperatures depended on the rearing temperatures, whereas Tsukada (*26*) determined that growth rates and heat and cold tolerances were affected by prenatal thermal history.

Guppies tolerate a wide range of salinities from fresh to full-strength sea water. Gibson and Hirst (*23*) demonstrated growth to be faster in 1/4 (8°/₀₀ or 8 parts per thousand) sea water than in fresh water. Similar rearing experiments with stock of different origin (*27*) show no difference in mean growth between fresh and 1/4 sea water. Considering the unknown history of these stocks, such differences are likely a result of adaptation for different original environments. In 1949, Haskins and Haskins (*28*) mentioned that populations of the guppy in lagoons and lower river courses may represent a physiologically distinct race from those of upland fresh waters.

I have maintained guppies at pH 5.5 to 8.5. Although little differences were noted in growth over this range, fish did best between pH 7.0 and 8.0.

Photoperiod has little effect on growth rate of young guppies as long as the ration level is held constant. Photoperiods of 24 hours light, 12 hours light/12 hours dark, and 1 hour light/23 hours dark showed no difference in mean growth after several months under these conditions (*24*). The reproductive capacity of female guppies, however, is affected by the photoperiod (*3*). Under natural photoperiods, the shortest intervals between broods and the most reproduction overall occurred in late spring and early summer months. For routine maintenance of stock, an artificial photoperiod of 16 hours light/8 hours dark or a natural photoperiod are both adequate.

Although the guppy can tolerate high population densities, much experimental work revealed growth and fertility as inversely correlated with population density and available living space (*9, 29–33*). Population dynamics in a restricted water volume is typified by the attainment of an asymptote or maximum number which depends on the amount of space even when food is in excess (*7, 29, 34*). Cannibalism of infants by adults is important in this population regulation. At high densities, cannibalistic behavior increases (*35*). One can minimize such a phenomenon in the culture of guppies simply by rearing young in ample space and by establishing matings that involve 1 male with 1 to several females. The fecundity of the guppy is such that matings involving many females are not necessary.

The guppy in its native environment as well as in the laboratory will feed on a wide range of food items. Green et al. (*36, 37*), studying the feeding habits of guppies introduced in Indonesia, showed the diets to be based on zooplankton and phytoplankton in some lakes and on benthic invertebrates, algae, and organic debris in others. In their native streams of Trinidad, the guppy was mainly a benthic feeder, e.g., algae, organic debris, and benthic invertebrates (*38*). Laboratory studies by these same authors on growth rate and maturation time on diets of live *Daphnia,* commercial flake food (Tetramin), and algae showed best growth and earliest times of maturity with the *Daphnia* diet. Fish on the Tetramin diet did almost as well as those fed *Daphnia.* It has been my experience that a mixed diet of commercial flake food and newly hatched brine shrimp (*Artemia*) or live *Daphnia* has proved adequate; growth rates and times of maturation were comparable to the *Daphnia* diet of Dussault and Kramer (*38*).

A laboratory-prepared food (liver-cereal paste) as described by Gordon (*39*) is also an excellent diet and may prove a less expensive alternative for the maintenance of a large number of fish. The amount of food fed to the fish is, of course, important. Guppies will grow faster and larger when given more food, if other factors, such as space and numbers, are not restricted. Too much food is detrimental only when it remains uneaten and fouls the water.

Other than factors, such as temperature, salinity, and pH, perhaps the most important environmental aspect involves the degree of conditioning of the water. Winge (*14*) mentioned that old aquarium water, yellow with humus and uric byproducts is ideal. Although water of such characteristics is not necessary for proper growth and maintenance of the guppy, the importance of aged and conditioned water cannot be ignored. At the Genetics Laboratory of the New York Aquarium, water is seldom discarded but reused for many years. Ordinary tap water may contain ions or ionic compositions toxic to fish. To help remove some toxic components (i.e., chlorine), one can "age" tap water by allowing it to sit and aerate for a time (days to weeks) before use, but often this is not enough. The conditioning of water by the use of aquatic plants or other (unimportant) fish can complete the adjustment of its ionic composition and bring mortality down to zero when fish are transferred to new quarters. When new aquaria need to be filled, conditioned water can be taken from older aquaria (not more than 25% at a time) and that water replaced by aged (at least several days) tap water. Water lost by evaporation can also be replaced in the same manner.

In housing and routine maintenance, a balance must be achieved between maximum economy of cost and space and growth and reproduction of the fish. The ideal situation results in the maximum number of fish in a given space without drastic reduction of fecundity and growth. Winge (*14*) mentioned that for matings (pairings) he used 2-liter preserving jars. He also used the containers for batches of 8–12 males or 3–5 females. I have used comparable densities, although for maximum growth a larger volume-to-fish ratio is desirable. Table 1 shows growth of newborn guppies reared at a density of 1 fish/2 liters. These fish reared at 24° C with unrestricted rations (Tetramin and newly hatched brine shrimp) had growth rates that can be considered maximum and maturation times near minimum for this species.

Although some strains of guppies grow faster in saline waters, the ease of maintaining a fresh water system over a salt system makes fresh water the better overall medium for most needs. Filtration and aeration are not usually necessary if densities are kept low enough and fish are not overfed. Aquatic plants are desirable because they provide a good hiding place for young and serve as substrates for algae and small invertebrates on which the fish can forage as a supplementary food source. A list of suitable fresh water aquarium plants with their descriptions and requirements are given by Gordon (*39*).

TABLE 1.—*Standard length (mm) and time at first sign of maturation (anal fin differentiation in first male) in 3 broods of* P. reticulata *reared at densities of 1 fish/2 liters with unrestricted rations*

Brood No.	Days	Females			Males		
		n	$\bar{x}$	SD	*n*	$\bar{x}$	SD
1	21	11	13.8	.56	10	14.1	.50
2	35	5	15.9	.42	5	16.1	.42
3	30	8	15.1	.42	7	15.1	.35

The young should be separated from the parents as soon as possible after birth. For careful maintenance of strains, fish must be sexed before they reach maturity. Because the females can retain sperm, the pedigree of her offspring can never be ascertained after insemination by an unknown male. Many workers separate the sexes as they begin to show signs of maturity (puberty spot in females and anal fin differentiation in males). One can also sex young before this time by looking at the relative position of the anal fin to the dorsal (*40*). In juvenile males, the anal fin is placed more anteriorly than it is in the female. The difference is subtle, but, with practice, prematuration sexing in this way can be 100% accurate.

Guppies are for the most part extremely hardy and resistant to most diseases which occur in the cultivation of fresh water fishes. General stresses, such as greatly fluctuating temperatures, overcrowding, overfeeding (fouling the water), and inadequate diet can reduce their resistance to various infections. These stresses should occur rarely if at all under proper maintenance, and the disease-free culture of these animals should be expected. Perhaps the most common infection seen in fresh water fishes and which will infect the guppy is the parasitic ciliate *Ichthyophthirius*. Fungal infections (usually secondary to an injury or other infection), bacterial infections, and various parasitic infections may also occasionally break out in the culturing of these fish. For identification and treatment of these, one should refer to any one of the many texts on fish diseases. Treatment for most of the common infections is easy, and several methods and drugs are readily available from sources in the aquarium fish trade or market.

PAST USE IN CARCINOGENICITY TESTING

The specific use of the guppy in cancer research is not as extensive as for other areas of biological research. Early reports deal with little more than the occurrence and description of spontaneous neoplasms [*see* (*41, 42*) for review]. However, recent reports have dealt with more critical dose–response studies. Work by Sato et al. (*43*), Pliss and Khudoley (*44*), Scherf (*45*), and Simon and associates (*46*) show the carcinogenicity of different substances on the guppy (table 2). Of the 10 known carcinogens tested in the above work, 7 have had carcinogenic effects. Most result in tumors of the liver, although those of the kidneys, intestine, and mesentery were also observed.

Differences in dose methodology, strength, and duration among these reports make comparisons between carcinogenic agents difficult. Sato et al. (*43*), when testing the addition of aflatoxin B_1, dimethylnitrosamine, and *N*-2-fluorenylacetamide to the diet, generally found hepatic tumors only after 11 months. Pliss and Khudoley (*44*), using several treatment methods and dosages with 9 car-

TABLE 2.—*Summary outline of carcinogens tested on* P. reticulata *and their actions*

Agent	Action	Means of exposure	References
Aflatoxin B_1	Hepatic tumors	Dietary	(*43*)
N-2-Fluorenylacetamide	" "	"	(*43, 44*)
7,12-Dimethylbenz[*a*]anthracene	No tumors	im and ip injections, skin applications, and dietary	(*44*)
3-Methylcholanthrene	" "	" " " " " " " "	"
Benzidine	" "	Dietary	"
o-Aminoazotoluene	Hepatic tumors	"	"
4-Dimethylaminoazobenzene	" "	"	"
Dimethylnitrosamine	" "	Dietary, added to water	(*43, 44*)
Diethylnitrosamine	" "	Added to water	(*43, 44, 46*)
	No tumors		(*45*)
Nitrosomorpholine	Hepatic, intestinal, and mesenchymal tumors	Added to water	(*44*)

cinogens, showed tumor induction by 6 of these. Their tumor induction times ranged from less than 3 to over 8 months, depending on the carcinogen tested, its route of application, and the dose strength. Of the exposure methods used, the authors claim that the most effective procedure is dissolving the carcinogen directly into the water. Topical application, im and ip injections, implantations, and diet supplementation were also used and found not as effective in tumor induction. Different carcinogens were tested with these methods, and the lesser response may well be a result of the relative carcinogenicity of the compounds tested and not the dosage or route of administration.

The carcinogenicity of nitrosamines has been demonstrated by numerous investigators (*43, 46*). In his experiments with diethylnitrosamine, Scherf (*45*) found no tumor induction after 7 months. His failure to detect any such effects with comparable doses and methodology does raise some questions concerning test consistencies. The lack of tumor induction may be a result of different experimental procedures or in differences of fish stock used.

The failure of the polycyclic aromatic hydrocarbons, 7,12-dimethylbenz[*a*]anthracene and 3-methylcholanthrene, to induce tumors in guppies is in agreement with the results of Rosenbauer (*47*), who tested several other species of fish. Pliss and Khudoley (*44*) believe this lack of tumor development may be a result of differing metabolic pathways between fish and other vertebrates because enzymes which convert polynuclear aromatic hydrocarbons into their carcinogenic metabolites may be low in fish. However, their explanation would not hold for all fish species based on the experience of Schultz and Schultz (*48*).

DISCUSSION

The advantages of the guppy for carcinogenicity testing are its 1) general hardiness with resulting ease of maintenance, 2) fecundity, 3) fast growth and short generation time, 4) small size with resulting small space requirements, 5) low maintenance cost, and 6) general availability.

In toxicity assays, too hardy a test organism is generally not recommended because this often results in reduced sensitivity to a given substance, whereas in carcinogenicity testing, this can become an advantage. Many carcinogens are also general toxicants, often producing tissue damage rather then neoplasms. Because tumor induction follows a postexposure latency, it is necessary for the fish to live through the general toxic effects of the carcinogen. The fact that death due to general toxic effects is important even in the guppy is realized by the high mortality even before the first tumors were observed, as reported by Sato et al. (*43*) and Pliss and Khudoley (*44*).

The ease of maintaining guppies is equalled by only a handful of the more common aquarium fish. The zebra fish (*Brachydanio rerio*) and the medaka (*Oryzias latipes*) are of comparable size and hardiness. However, both lay eggs, and the extra steps necessary for egg collection, hatching, and rearing of the comparatively smaller young make the cultivation of these species slightly more difficult and time-consuming. The introduction of the guppy, one of the most readily available of small aquarium fish throughout the world today, for mosquito control has resulted in a large source of introduced "natural" populations. Its popularity in the aquarium fish trade makes it one of the most inexpensive and readily obtainable. This popularity of the guppy for mosquito control and the pet trade has resulted in problems concerning stock origins. In its native populations, the guppy offers a large degree of variability as shown by the number of color patterns and fin shapes which have been described. This natural variability, under the artificial selection of fish breeders or under the exotic natural selection pressures of a foreign environment, is constantly being increased. The source of many guppies in research today is ultimately the pet trade. Examination of the evolutionary, ecological, and behavioral problems of natural guppy populations comprises much of the current research on this fish, in which stock from known wild origins is used. The problem of multiple potential sources of fish stock is that we do not have a good genetic history of these fish. This is not an insurmountable problem because the differences between strains are often insignificant. However, differences in stock origin may account for some contradictory reports concerning growth response and sensitivity to environmental variables. Information gained from a stock regardless of the origin of the strain can be more useful if the stocks are maintained and available for future work. Consistency of stock use is more important than the ultimate origin of the stock. The establishment of carefully maintained inbred strains for cancer research is the best likely solution to this problem.

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Use of Aquarium Fish, *Danio rerio* and *Poecilia reticulata*, as Test Species for Evaluation of Nitrosamine Carcinogenicity[1]

Veniamin V. Khudoley[2, 3]

ABSTRACT—The carcinogenic effects of various doses of dimethylnitrosamine (DMNA), diethylnitrosamine (DENA), and nitrosomorpholine (NM), as well as the results of varying temperature and length of exposure to these carcinogens, were studied in short-term (20–21 wk) experiments in aquarium fish (820 *Danio rerio* and 944 *Poecilia reticulata*). All nitroso compounds induced liver tumors (hepatocellular carcinomas and adenomas, cholangiomas, and cholangiocarcinomas) and esophageal papillomas. Exposure to NM also induced intestinal adenocarcinomas in *D. rerio*. At a conventional temperature (22° ± 1° C), tumors were induced in 76% of the *D. rerio* in an average of 13.8 weeks following an initial 8-week exposure to 100 ppm DMNA and in 22% of *P. reticulata* in an average of 10.9 weeks. The DENA exposure resulted in tumors in 61.5% of *Danio* by 15 weeks and in 29% of *Poecilia* in 15.5 weeks. Under the same conditions, 500 ppm NM induced tumors in 78% of the zebra fish in 15.6 weeks and in 75% of the guppies after 12.3 weeks. When exposure time was shortened to 2 weeks, the dosage was decreased, or if the temperature was reduced to 17° ± 1° C, the tumor incidence dropped and the latency increased. An increase in temperature to 27° ± 1° C resulted in a 72–89% tumor incidence and shortened the latency to 11.3–14.1 weeks. Thus the modulation of our experimental conditions to maximize tumor incidence and minimize mean latency permitted the use of the aquarium fish *D. rerio* and *P. reticulata* for the screening of carcinogenic nitrosamines. — Natl Cancer Inst Monogr 65: 65-70, 1984.

As initially stated by Stanton (*1*) and later confirmed by numerous investigators (*2–6*), the marked sensitivity of aquarium fish to tumor induction by nitrosamines permits the use of these animals for carcinogenicity studies. Moreover, aquarium fish possess a number of advantages as test animals for the evaluation of the carcinogenicity of these compounds; these advantages have been summarized by Khudoley (*7*) and by Matsushima and Sugimura (*8*).

Here I present data from several short-term investigations on the induction of tumors in 2 species of aquarium fish, the zebra fish *Danio rerio* and the guppy *Poecilia reticulata*, by exposure to different doses of various nitrosamines. The influence of exposure time and temperature on tumor induction was also assessed.

MATERIALS AND METHODS

Fish.—The 820 zebra fish *D. rerio* and 944 guppies *P. reticulata* were obtained from a commercial breeder. We used 10- to 12-month-old adult fish of both sexes. The animals (40–50/group) were kept in 20 liters of dechlorinated tap water with aeration 8 hours a day and illumination by daylight and luminescent lighting each for 12 hours a day. During the studies, the fish were given fly larvae and dry vitamin-enriched food (Tetramin) six times a week.

Carcinogens.—The DMNA, DENA, and NM were synthesized by the Laboratory of Organic Chemistry (N. N. Petrov Research Institute of Oncology, Leningrad). After the identity and purity of these compounds were ascertained by spectroscopy, the carcinogens were added directly to the water in the aquarium.

Experiments.—The design of the experiments is represented in table 1. After exposure for 2 or 8 weeks to the various nitrosamines in the first 3 series of experiments, all survivors were placed in fresh water without carcinogens and held there until the end of the experiment (20 wk for zebra fish and 21 wk for guppies). The water temperature was 17°, 22°, or 27° C according to the experimental group and was always held constant (±1° C) by the use of a thermoregulator.

In the fourth series of experiments, we assessed the influence of the water temperature on untreated fish. The animals were maintained at a high (27° ± 1° C) or low 17° ± 1° C) temperature for 20–21 weeks and then switched to 22° ± 1° C water until the study was terminated at 52 weeks. Throughout the experiments, the fish were examined carefully for changes in appearance and behavior. Some fish died during this period as a result of lethal intoxication, parasitic invasion, tumors, or other causes. All dead fish and survivors were autopsied and the visceral organs examined grossly. For histological examinations, whole fish were fixed in Bouin's fluid. The material was fixed for 48–72 hours and then embedded in paraffin. Cross and frontal sections were stained with H & E and van Gieson's stains. All the results were treated statistically by the chi-square method for tumor incidence and the *t*-test for comparison of the average latent periods.[4]

ABBREVIATIONS: DMNA = dimethylnitrosamine; DENA = diethylnitrosamine; NM = nitrosomorpholine; H & E = hematoxylin and eosin.

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[4] Latency is defined as an interval or period between the beginning of exposure to the carcinogen and death with tumor or by killing.

TABLE 1.—*Scheme of experiments*[a]

Carcinogen	Series and group	No. of fish	Species	Dose, ppm	Time of exposure, wk	Temperature, degree C
DMNA	1.1	50	Zebra	20	8	22 ± 1
	1.2	50	"	50	8	22 ± 1
	1.3	50	"	100	8	22 ± 1
	1.4	40	"	100	2	22 ± 1
	1.5	50	"	100	8	17 ± 1
	1.6	50	"	100	8	27 ± 1
	1.7	50	Guppy	100	8	22 ± 1
	1.8	49	"	100	2	22 ± 1
DENA	2.1	120	Zebra	100	8	22 ± 1
	2.2	40	"	100	2	22 ± 1
	2.3	50	Guppy	33	8	22 ± 1
	2.4	50	"	50	8	22 ± 1
	2.5	50	"	100	8	22 ± 1
	2.6	50	"	100	2	22 ± 1
	2.7	45	"	100	8	17 ± 1
	2.8	50	"	100	8	27 ± 1
NM	3.1	40	Zebra	300	8	22 ± 1
	3.2	40	"	500	8	22 ± 1
	3.3	40	"	300	8	17 ± 1
	3.4	40	"	300	8	27 ± 1
	3.5	55	Guppy	250	8	22 ± 1
	3.6	50	"	500	8	22 ± 1
	3.7	50	"	500	2	22 ± 1
	3.8	50	"	250	8	17 ± 1
	3.9	50	"	250	8	27 ± 1
Control	4.1	115	Zebra			22 ± 1
	4.2	40	"			17 ± 1[b]
	4.3	55	"			27 ± 1[b]
	4.4	195	Guppy			22 ± 1
	4.5	50	"			17 ± 1[c]
	4.6	50	"			27 ± 1[c]

[a] The period of observation in experiments on zebra fish was 20 wk; for guppies, it was 21 wk (in group 1.7, 17 wk); and in controls (zebra fish and guppies), it was 52 wk.

[b] After 20 wk, the temperature was 22° ± 1° C.

[c] After 21 wk, it was 22° ± 1° C.

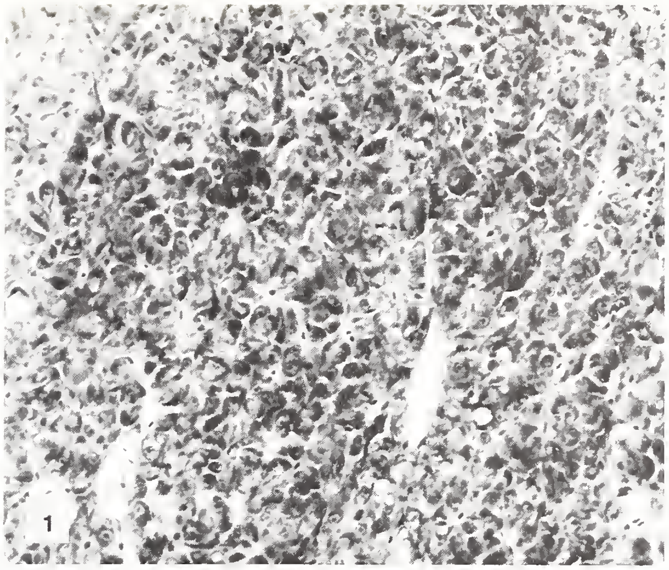
RESULTS

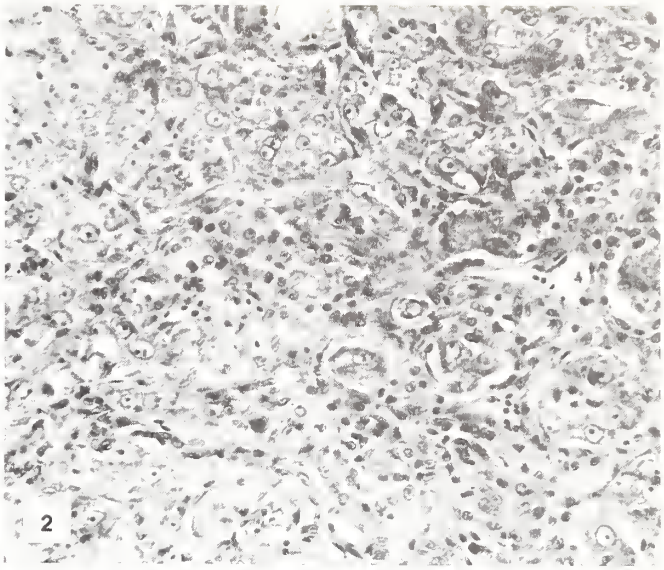
Changes in behavior, including decreased motor activity, were evident within 2–3 weeks from the beginning of exposure. Microscopic examination of the liver in this period revealed rearrangement of the organ's structure, focal necrosis, fatty dystrophy of hepatic cells, and hyperplasia with the formation of numerous or conspicuous protuberances composed mainly of basophilic hepatocytes. Five to 7 weeks later, focal proliferation of the bile duct epithelium was seen. Associated with these preneoplastic lesions were tumors already described (*9, 10*).

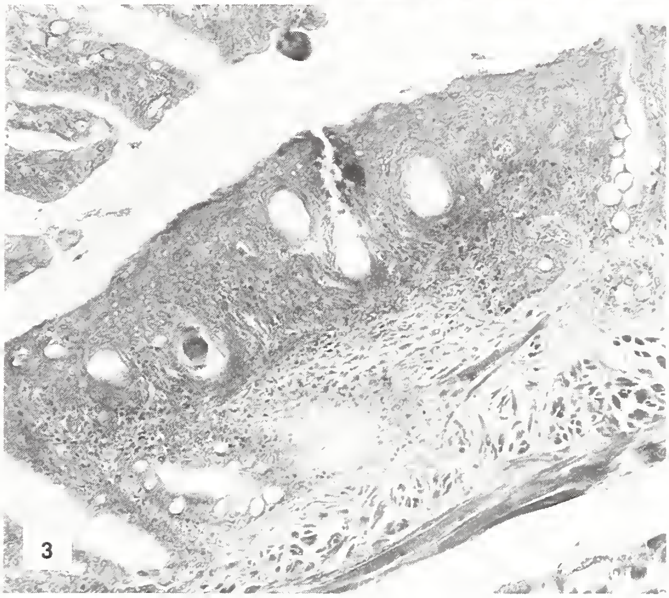
Most of the liver neoplasms were hepatocellular carcinomas comprised of various differentiated cells (fig. 1); the hepatocytes appeared pleomorphic with marked polychromism and often contained atypical mitoses. The tumor foci were polycentric, and invasive growth into adjacent tissue was apparent. Hepatocellular adenomas developed from nodules comprised of basophilic cells and were tubular or trabecular. Epithelial proliferation of the bile ducts was evident; single or multiple cystocholangiomas of various sizes subsequently developed from these proliferative lesions. Cholangiocarcinomas containing pleomorphic and polychromic cells and exhibiting numerous mitotic figures and invasive growth were sometimes seen (fig. 2). Cholangiofibrosis often accompanied cholangiolar tumors.

In the esophagus of fish treated with nitrosamines, we saw evidence of dysplastic areas as well as multiple typical papillomas consisting of basophilic epithelial cells with large dark nuclei (fig. 3). We noted all these tumor types in the 4 series of experiments. In addition, NM-treated zebra fish developed malignant intestinal adenocarcinomas constricting the intestinal lumen (fig. 4). Areas with distinct desmoplastic growth and occasional large amounts of mucin were seen. The cells, pleomorphic with a high mitotic activity, infiltrated all layers of the intestinal wall. However, we observed no metastases into other organs.

Toxic lesions represented by focal necrosis were in the kidneys of nitrosamine-treated fish. Subsequently, inflammatory processes within the renal tubules and atrophic lesions in the parenchyma appeared. Results of these experiments are summarized in tables 2–4.







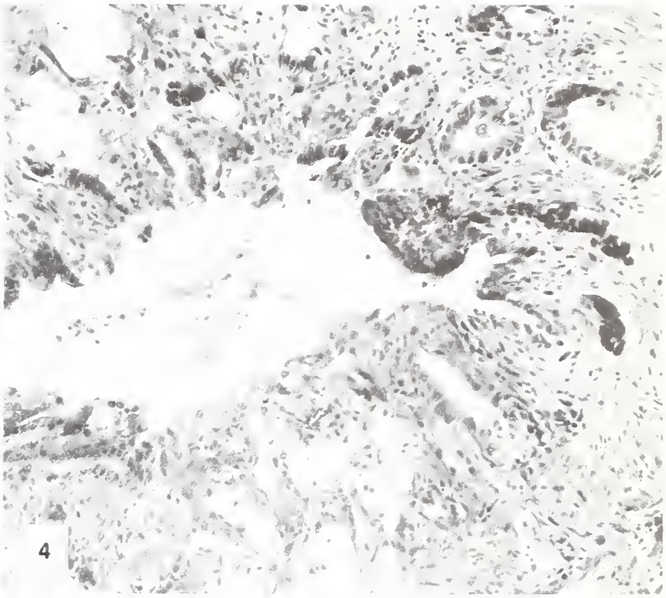


FIGURE 1.—Hepatocellular cancer, group 1.3 (100 ppm DMNA, *D. rerio*), 10 wk. H & E. × 440
FIGURE 2.—Cholangiocarcinoma, group 2.5 (100 ppm DENA, *P. reticulata*), 11 wk. H & E. × 440
FIGURE 3.—Papilloma of the esophagus, group 2.5 (100 ppm DENA. *P. reticulata*), 8.5 wk. H & E. × 120
FIGURE 4.—Intestinal adenocarcinoma, group 3.2 (500 ppm NM, *D. rerio*), 12 wk. H & E. × 230

Experiment 1

The incidence of tumors (expressed as number of fish with tumors/number of fish surviving to the first tumor) in zebra fish increased from 31 to 76% in animals treated with increasing doses from 20 to 100 ppm DMNA ($P<0.01$). The average latent period until tumor development in fish exposed to 20 and 100 ppm was 18.2 ± 0.5 and 13.5 ± 0.6 weeks, respectively ($P<0.05$). By reducing the exposure to DMNA from 8 to 2 weeks, we found that a longer average latency resulted in lower numbers of tumors (groups 1.4 vs. 1.3: $P<0.01$). In addition, the temperature influenced tumor frequency and latency. In zebra fish kept in cold water (17° ± 1° C), the tumor incidence decreased to 38%, whereas in those fish held at 27° ± 1° C, this incidence was 88% ($P<0.01$). The average latent periods were 16.6 ± 0.8 and 11.3 ± 0.6 weeks, respectively ($P<0.05$). The number of tumors per tumor-bearing fish varied from 1 to 1.25; these differences were not statistically significant.

The first tumor was observed after 5 weeks in guppy group 1.7 (8-wk exposure to DMNA) and after 17 weeks in group 1.8 (2-wk exposure), and the overall tumor incidences were 12.5 and 22%, respectively.

In this study, 142 of 148 neoplasms were in the liver, with 116 hepatocellular and 26 cholangiolar in origin. In those groups of zebra fish held at a higher temperature and exposed to higher levels of DMNA, 6 esophageal neoplasms occurred.

TABLE 2.—*Tumor induction by DMNA in aquarium fish, groups 1.1–1.8*

Group No.	Time of first tumor appearance, wk	Average latency, wk	Tumor incidence		Total No. of tumors	Tumors		
						Liver		
			No.[a]	Percent		Hepatocellular	Cholangiocellular	Esophageal
1.1	13	18.2±0.5	13/42	31	13	12	1	
1.2	9	16.1±0.7[b]	25/47	53	26	18	7	1
1.3	8	13.8±0.6[b]	35/46[c]	76	40	30	8	2
1.4	17.5	19.3±0.3[d]	8/31[d]	26	8	8		
1.5	11.5	16.6±0.8[d]	11/29[d]	38	11	11		
1.6	5.5	11.3±0.6[d]	36/41	88	43	31	9	3
1.7	5	10.9±2.5	4/18	22	5	4	1	
1.8	17	19.0±2.0[e]	2/16	12.5	2	2		

[a] Values indicate No. of fish with tumors/No. of fish survived to the first tumor observation.

[b] Differences with group 1.1 are significant ($P<0.05$).

[c] Differences with group 1.1 are significant ($P<0.01$).

[d] Differences with group 1.3 are significant ($P<0.01$).

[e] Differences with group 1.7 are significant ($P<0.05$).

TABLE 3.—*Tumor induction by DENA in aquarium fish, groups 2.1–2.8*

Group No.	Time of first tumor appearance, wk	Average latency, wk	Tumor incidence		Total No. of tumors	Tumors		
						Liver		
			No.[a]	Percent		Hepatocellular	Cholangiocellular	Esophageal
2.1	8	15.0±0.4	68/111	61.5	76	51	22	3
2.2	15.5	17.3±0.9[b]	6/27[c]	22	6	5	1	
2.3	12.5	18.2±1.6	5/38	13	7	5	2	
2.4	12.5	17.1±1.2	6/41	14.5	7	5	2	
2.5	6	15.5±1.3	12/42	29	16	10	3	3
2.6	16.5	19.0±0.6[d]	8/46	17.5	8	7		1
2.7	17.5	19.1±0.4[d]	10/33	30.5	10	9		1
2.8	6.5	12.7±0.7	31/43[e]	72	39	29	6	4

[a] Values indicate No. of fish with tumors/No. of fish survived to the first tumor observation.

[b] Differences with group 2.1 are significant ($P<0.05$).

[c] Differences with group 2.1 are significant ($P<0.01$).

[d] Differences with group 2.5 are significant ($P<0.01$).

[e] Differences with group 2.5 are significant ($P<0.01$).

Experiment 2

In zebra fish exposed to 100 ppm DMNA for 2 or 8 weeks, tumors developed after 17.3±0.09 and 15.0±0.4 weeks ($P<0.05$) in 22 and 61.5% of the fish, respectively ($P<0.01$). No significant differences were noted in the tumor incidences or latent periods of guppies exposed to different doses of DENA. However, in group 2.8, a significantly increased incidence of tumors was evident (group 2.8 vs. 2.5: $P<0.01$); these fish had been exposed to the highest concentration of DENA and held at the highest water temperature (27° C). On the other hand, a reduction in latency was noted when we compared groups 2.5 and 2.6 ($P<0.05$), and 2.5, 2.7, and 2.8 ($P<0.05$). Therefore, these data showed a marked dependence of carcinogenic response on temperature and length of exposure to the compound. As in the previous experiment, liver neoplasms predominated (157 of 169), with 121 fish exhibiting hepatocellular and 36 having cholangiolar tumors. Twelve fish developed esophageal tumors.

Experiment 3

The first tumor induced by NM in zebra fish was found after 7 weeks. A significantly increased number of tumors (78%) occurred in the high-dose (500 ppm) group compared with the low-dose (250 ppm) group, in which 21.5% of fish developed tumors ($P<0.01$). The tumor incidence among fish exposed to 300 ppm NM and held at 27° C increased significantly ($P<0.01$) when compared with fish exposed to the same dosage but held at 22° C (82.5% in group 3.4 vs. 21.5% in group 3.1). However, the average latent periods in all these groups were practically the same, except in group 3.3 in which fish were held at 17° C. Only 1 tumor-bearing fish was found after 20 weeks.

The first tumor in a guppy appeared in 6 weeks. Group differences in tumor frequency were not evident; moreover,

TABLE 4.—*Tumor induction by NM in aquarium fish, groups 3.1–3.9*

Group No.	Time of first tumor appearance, wk	Average latency, wk	Tumor incidence		Total No. of tumors	Tumors			
						Liver		Esophageal	Intestinal
			No.[a]	Percent		Hepatocellular	Cholangiocellular		
3.1	9.5	15.3 ± 1.9	6/28	21.5	6	5			1
3.2	10.5	15.6 ± 1.4	7/9[b]	78	9	5	2		2
3.3	20	20[c]	1/18	5.5	1		1		
3.4	7	14.1 ± 1.2	14/17[b]	82.5	18	13	2		3
3.5	11.5	16.7 ± 1.0[d]	10/16	62.5	10	9	1		
3.6	8.5	12.3 ± 2.0	3/4	75	4	3		1	
3.7	14.5	18.3 ± 2.0[d]	3/8	37.5	3	2	1		
3.8	13.5	18.0 ± 0.9	8/15	53.5	8	8			
3.9	6	13.0 ± 1.0[e]	8/9	89	11	7	2	2	

[a] Values indicate No. of fish with tumors/No. of fish survived to the first tumor observation.

[b] Differences with group 3.1 are significant ($P<0.01$).

[c] Differences with group 3.1 are significant ($P<0.05$).

[d] Differences with group 3.6 are significant ($P<0.05$).

[e] Differences with group 3.5 are significant ($P<0.01$).

the length of the latent period depended strictly on dosage (groups 3.5 vs. 3.6: $P<0.05$), exposure time (groups 3.6 vs. 3.7: $P<0.05$), and temperature (groups 3.5 vs. 3.9: $P<0.01$). Not only were tumors found in the livers (61 fish) and esophagus (3 fish), but intestinal adenocarcinomas also were observed in 6 zebra fish.

Experiment 4

No neoplastic or preneoplastic lesions were noted in the long-term (52 wk) observations on control (untreated) fish.

DISCUSSION

Nitroso compounds possess a broad range of biological activity, inasmuch as they are known to induce tumors in 39 animal species including 5 fish species (*11*). Despite the fact that the carcinogenicity of these substances for humans has not been established, they should be considered as potentially hazardous environmental pollutants (*12*). That is why it is especially important for one to establish short-term, direct biological methods for the evaluation of the carcinogenic properties of nitrosamines. Aquarium fish appear to be suitable animals for this purpose, and investigations in our laboratory confirmed the high sensitivity of aquarium fish *D. rerio* and *P. reticulata* to the action of the carcinogens DMNA, DENA, and NM.

A measure of the comparative sensitivity of animals to various carcinogens may be served by the Iball index: percent tumor incidence/number of days in latent period × 100. For example, in the experiments with DMNA, the index values for groups 1.3 and 1.7 are 79 and 29, respectively ($P<0.01$). Among DENA-treated fish, these values were 59 and 27 in groups 2.1 and 2.5, respectively ($P<0.01$). This assessment suggests that, despite the absence of any statistical differences in tumor frequency and length of the latent period, *D. rerio* are obviously more sensitive than *P. reticulata* to the action of DMNA and DENA. At the same time, we could compare the carcinogenic activity of nitroso compounds using equimolar concentrations of tested agents when the temperature and duration of exposure are similar.

As was demonstrated in these studies, an increase in carcinogen concentration led to a higher tumor incidence and a decreased average latency. We noted a similar tendency when either the exposure time was lengthened or the water temperature was raised. Contrary to the studies by Kyono (*13, 14*) in which medakas (*Oryzias latipes*), kept at low temperatures and exposed to DENA, did not develop tumors during a 12-week observation period, we induced neoplastic lesions in guppies and zebra fish held at 17° C. This result was probably dependent on the longer observation period rather than on a higher sensitivity of guppies and zebra fish to the actions of carcinogens.

Nitroso compounds induce neoplasms at sites in *D. rerio* and *P. reticulata* in which spontaneous tumors are rarely found, i.e., the esophagus, intestine, and especially, the liver (*7, 15, 16*). These tissues are obviously target sites for the carcinogenic action of nitroso compounds.

Thus the relatively short latent period (compared with the period in mammals) and high incidence of neoplasms that are easily modulated by experimental conditions allow for the use of the aquarium fish *D. rerio* and *P. reticulata* in the screening of carcinogenic nitrosamines.

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Carcinogenesis Studies on Guppies[1]

Károly Simon *and* Károly Lapis[2, 3]

ABSTRACT—Guppies (*Lebistes reticulatus*) were used as models for the screening of potentially carcinogenic substances. These fish, a simple, readily accessible type, are sensitive toward carcinogens. Using this model, we performed studies on the morphological effects of a few water-soluble chemicals. Guppies treated with diethylnitrosamine were used as positive controls. Changes were noted in the livers, and a few specific lesions occurred in their gastrointestinal tracts. Studies were also conducted with 10 other similar chemicals found in the human environment that have potential use in chemotherapy. Of these, the carcinogenic effect of *N,N'*-dinitrosopiperazine on fish was demonstrated. Some researchers think this compound is a derivative of the pesticide Triforine. Furthermore, 2 of the 10 chemicals intended for use in therapy proved to be potential carcinogens. Because these experiments with water-soluble substances are inexpensive and the test periods are short, the use of fish is considered more advantageous than is the use of rodents. — Natl Cancer Inst Monogr 65: 71-81, 1984.

Assessment of the potential carcinogenesis of chemical substances found in the human environment is a difficult task. Because of the vast number of existing chemicals, the currently accepted bioassay protocol in which 2 rodent species are used is insufficient for testing so many compounds. Researchers are now attempting to establish new experimental systems that can give relevant information in less time (*1*). Among the short-term heterogeneous tests, the most significant are the in vitro mutagenesis assays (*2, 3*). By contrast, the objective of many investigators is to establish useful short-term in vivo testing systems for carcinogenesis. According to the literature (*4, 5*), some compounds are carcinogenic in both rodents and fish. Sato and his co-workers (*6*) have also suggested that fish are suitable for the screening of possible carcinogens.

At this Institute, our aim was to develop fish as a test species for assessing the possible carcinogenic effects of certain chemicals that are present in the human environment and are intended for therapeutic use. According to previous studies, the time needed for tumor induction is less in fish than in rodents; moreover, the tests are less expensive (*7*). Therefore, our objective was to use a fish species readily accessible and sensitive to the effect of carcinogens and to elucidate the most effective means of exposure.

MATERIALS AND METHODS

Inbred, 2- to 5-month-old, randomly selected male and female guppies, *Lebistes reticulatus*, were used. The fish were obtained from a hatchery (MÉM Plant Protection and Agrochemical Center, Hydrobiological Laboratory, Százhalombatta, Hungary). The fish were held in 25- and 50-liter fish tanks containing dechlorinated tap water that was circulated through a nylon-wool filter. An external compressor was provided for aeration. The temperature of the aquarium water was maintained at 22°-25° C, and a 12-hour light-and-dark illumination cycle was provided. In the experiments, the fish received Tetramin (Tetra Werke, Melle, Federal Republic of Germany) ad libitum. Initially, we used DENA, which, according to previous studies (*8-11*), proved to be carcinogenic in fish.

Having confirmed this effect in our studies (*12*), we then initiated experiments with compounds in which the carcinogenic effect had not been determined, particularly in fish. We studied the activity of *N,N'*-dinitrosopiperazine and 10 other coded compounds produced by a Hungarian pharmaceutical firm (EGYT-Pharmacochemical Works, Budapest) for future drug use (table 1). The compounds were soluble or made soluble by various procedures, i.e., by the addition of maleic acid or DMSO. Control fish treated only with these compounds showed no effects of toxicity. According to our measurements and to the data obtained from the drug firm, the chemicals examined were partly decomposed (about 20% within 1 mo). Therefore, we changed the water every second week, and the chemicals were added in appropriate concentrations.

Prior to the carcinogenicity studies, the toxicity of the compounds in the fish was determined; the data were evaluated by Litchfield-Wilcoxon analysis. Five doses of each compound were used ranging from 0.15 to 600 mg/liter. The number of animals per dose, the exposure time, and the LD50 determined from the 5 doses per compound are given in table 1.

We administered one-half and one-quarter dosages of the LD50 level. The exposure period was generally 2 months, after which a 1- to 2-month observation period followed.

ABBREVIATIONS: DENA = diethylnitrosamine; DMSO = dimethyl sulfoxide; LD50 = that dosage at which 50% of the animals die; H & E = hematoxylin and eosin; PAS = periodic acid-Schiff.

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[3] We are grateful to Dr. Eszter Ujhelyi of the Biochemical Laboratory of this Institute for performing the enzyme studies for our work.

TABLE 1.—*Lethality of compounds used in our experiments on carcinogenicity*

Name of chemical and code No.	No. of fish/dose	Dose range, mg/liter	LD50, mg/liter	Length of exposure, days
DENA	20	10, 25, 50, 100, 200	100	30
N,N′-Dinitrosopiperazine	20	25, 50, 100, 200, 400	170	30
1-Benzyl-4-(2′-pyridinecarbonyl)-piperazine-(E)-2-butenedioate (1:1); KL-137	5	37.5, 75, 150, 300, 600	170	30
L-(+)Threo-1-phenyl-2-isopropylaminopropandiol-1,3-hydrochloride; KL-138	5	37.5, 75, 150, 300, 600	50	30
D,L-(Erythro(+)alloerythro)-1-phenyl-2-[1′-methyl-2′-phenoxyethyl-(amino)]-1,3-propandiol hydrochloride; KL-139		4.7, 9.4, 18.7, 37.5, 75	10	30
2,3-Dimethoxy-8-aza-D-homo-9-β, 13-α, 14α-gona-1,3,5(10)-triene-12-one-(E)-oxime; KL-156		8.2, 16.5, 33, 66, 132	15	30
N-(α)-α-Methyl-*p*-chloro-benzhydryloxy-(ethyl)-hexamethylene imine hydrochloride; KL-157	5	0.15, 0.3, 0.6, 1.2, 2.5	0.8	30
1-(4-Chlorphenyl)-1-hydroxy-as-triazino-(6,1-α)-isochinolinium bromide; KL-169	5	15, 30, 60, 120, 240	90	4
2-Chloro-12H-12-[2-(4-methyl-1-piperazinyl)-ethyl]-dibenzo-[*d,g*]-[1,3,6]-dioxazocine-dihydrochloride; KL-170	5	7.5, 15, 30, 60, 120	15	4
2-Chloro-12H-dibenzo-[*d, g*]-[1,3,6]-dioxazocine-12-(*N,N,β*-trimethyl-propanamine) hydrochloride; KL-171	5	7.5, 15, 30, 60, 120	15	4
2-Chloro-12H-dibenzo-[*d,g*]-[1,3,6]-dioxazocine-12-(*N,N*-dimethyl-propanamine)-(Z)-butenedioate (1:1); KL-172	5	7.5, 15, 30, 60, 120	15	4
2-(E)-(phenylmethylene)-*O*-{2-[bis-(1-methylethyl)-amino]-ethyl}-cycloheptanone-(E)-exime-(E)-2-butenedioate (1:1); KL-173	5	7.5, 15, 30, 60, 120	30	4

(Sometimes this period lasted for 4 months.) During the observation periods, the fish were held in clean aquarium water, and the behavior of the fish was checked twice daily throughout the experiments.

In one study, the surviving fish were killed and prepared for histological examination at the end of the observation period (table 2). In a second, 5–10 fish were killed and prepared at fixed intervals according to the experimental group: Initially this was weekly, then monthly (tables 3, 4). Therefore, according to the protocol, 45–100 fish/dose were tested with DENA and *N,N′*-dinitrosopiperazine. We used 20–25 fish/dose of the compounds produced by the pharmaceutical firm.

For histopathological examination, the fish were immersed in 10% formol solution for fixation. Three to five sagittal sections were routinely prepared from the tissue embedded in paraffin. In certain fish in which the occurrence of a tumor was likely, we dissected the fish under a stereomicroscope before embedding. The sections were

TABLE 2.—*Frequency of liver tumors and precancerous alterations found at the end of the examination periods*[a]

Chemical used	Dose, mg/liter	No. of fish: Used in experiments	Survived to end of experiments	With preneoplastic lesions	With tumors
DENA	25	45	32	10	0
	50	95	54	13	13
KL-157[b]	0.12	25	17	7	0
	0.25	25	17	13	0
KL-170	2.5	20	12	4	0
	5	20	11	5	2

[a] Exposure period was 8 wk; observation period was 4 wk.

[b] The observation period was 8 wk.

TABLE 3.—*Preneoplastic lesions and tumors in livers of fish treated with DENA and* N,N′*-dinitrosopiperazine*[a]

Chemical used	Dose, mg/liter	No. of fish with preneoplastic lesions[b]	Observation period, wk	No. of fish with tumors[b]	Observation period, wk
N,N′-Dinitrosopiperazine	100	1/3	13		
	50	4/10	14	2/8	17
		2/8	17	2/5	20
		3/5	20	2/5	26
		1/5	23		
		3/5	26		
DENA	50	2/10	6	1/5	7
		1/5	7	2/10	8
		5/10	8	3/10	9
		5/9	9		
	25	1/10	6	1/5	7
		2/5	7	1/10	9
		4/10	9	2/3	26
		5/10	10		
		1/5	12		

[a] Total No. of fish was 100/dose. Fish were killed at fixed intervals.

[b] Values indicate No. of fish with preneoplastic lesions/No. of fish investigated in this group.

TABLE 4.—*Preneoplastic lesions and tumors in gastrointestinal tracts of fish treated with DENA and* N,N′*-dinitrosopiperazine*[a]

Chemical used	Dose, mg/liter	No. of fish with preneoplastic lesions[b]	Observation period, wk	No. of fish with tumors[b]	Observation period, wk
N,N′-Dinitrosopiperazine	100	2/10	4	1/10	4
	50	1/10	4	1/10	6
		2/10	6		
		1/10	14		
		2/5	23		
		1/5	26		
DENA	50	1/5	7	1/10	8
		1/9	9	1/9	9
	25	2/5	7		

[a] Total No. of fish was 100/dose. Fish were killed at fixed intervals.

[b] Values indicate No. of fish with preneoplastic lesions/No. of fish investigated in this group.

routinely stained with H & E, but with some fish, we followed the PAS, Goldner trichrome, Ziehl-Neelsen, and Gomori staining techniques. The fish that died during the experimental period were similarly prepared.

In separate studies, the livers of 20 untreated guppies (3 mo old) were examined, and their aryl hydrocarbon hydroxylase and glucose-6-phosphatase levels were determined and compared with Sprague-Dawley rats (obtained from the Laboratory Animal Center, Gödöllő, Hungary). We dissected the livers of the guppies for biochemical assay under a stereomicroscope and then pooled them. The total weight of the liver tissue from 20 guppies was 60 mg. The activities of both enzymes were determined according to the methods described by Diamond et al. (*13*) for the hydroxylase and Harper (*14*) for the phosphatase. Each sample was measured three times.

RESULTS

Untreated Fish

According to our data, the aryl hydrocarbon hydroxylase levels of the guppy and Sprague-Dawley rat livers were 1.75 and 20 U/mg protein, respectively, and the glucose-6-phosphatase levels were 15.08 ± 0.51 and 10.0 ± 1.0 μmol phosphorus/hour per mg protein, respectively.

As negative controls, untreated guppies were held under the same conditions as treated ones. During the course of our studies, we found neither preneoplastic nor neoplastic lesions in the negative controls.

The livers of untreated guppies exhibited a reddish-brown color with a smooth surface. The lobular pattern characteristic of mammalian liver was not evident. We noted sinusoids between the liver cell trabeculae that consisted of 2 rows of hepatocytes; the portal areas and the

FIGURE 1.—Histological picture of untreated guppy liver. Hepatocytes form trabeculae which are separated from each other by sinusoids; lobular structure is not apparent. Large veins are seen here lying irregularly throughout the parenchyma. H & E. × 400

central veins were irregular (fig. 1). In approximately 10% of the fish, the livers showed some fatty metamorphosis; however, in the 4- to 5-month-old fish, we found granulomas in the kidneys, gonads, and livers. We want to emphasize here the existence of foci described by Reichenbach and Klinke, consisting of mononuclear inflammatory cells, that develop as a result of mycobacterial infections (*15*). Sometimes, these could be mistaken for hyperplastic liver cell nodules. The demonstration of acid-fast *Mycobacterium* by the Ziehl-Neelsen staining technique aids in the differential diagnosis of these lesions.

Diethylnitrosamine-treated Fish

We treated our guppies serving as positive controls with DENA (Eastman Kodak Co., Rochester, N.Y.). On the basis of our toxicology data (*12*), doses of 25 mg and 50 mg/liter were administered. We found histological changes (summarized below) in the livers and gastrointestinal tracts of fish that died during the observation period as well as those killed at the scheduled times.

Early Degenerative or Necrotic Lesions

From the first and second weeks of treatment, we observed enlarged hepatocytes with foamy cytoplasm, as well as cells containing PAS- and Ziehl-Neelsen-positive granules, that contained ceroid. In addition to the above, focal necrosis of single hepatocytes or areas of hepatocytes were present, chiefly in sites close to the larger bile ducts. The necrotic cell fragments had undergone phagocytosis by mononuclear cells. The structure of the parenchyma was disorganized with collapsed sinusoids. In some instances, desquamation and necrosis of the bile duct epithelium could be observed.

Regenerative Signs

Beginning with the second week of treatment, we detected enlarged hepatocytes with 1–2 prominent nucleoli among the degenerated cells. In addition to the enlarged hepatocytes, cells having small oval nuclei were seen, usually close to portal areas. At the end of the third month (disregarding the slight increase in connective tissue around the bile ducts), no differences could be observed in the livers of some fish treated with DENA compared with the control groups.

Preneoplastic Lesions

Apart from the slight proliferation of the mesenchymal elements, a diffuse hyperplasia of the hepatocytes throughout the whole liver could be detected in approximately 20–30% of the DENA-treated fish (fig. 2). These hepatocytes showed slight or marked atypia with distinct nucleoli. The trabecular structure was altered, with the hepatocytes arranged in irregular groups, forming numerous hyper-

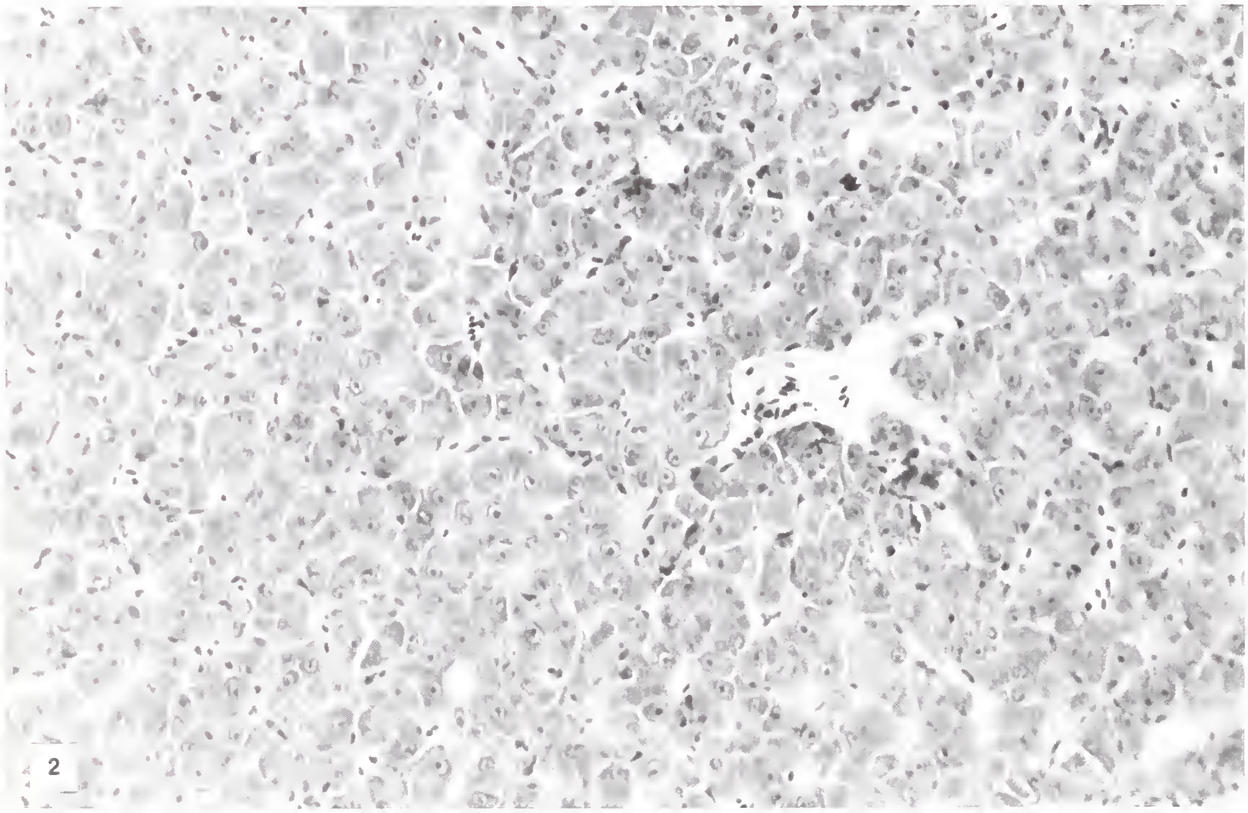


FIGURE 2.—Diffuse hyperplasia of the liver parenchyma. Trabecular structure has become disorganized. Parenchyma is characterized by the proliferation of cells showing a moderate degree of polymorphism and containing a prominent nucleolus. No signs of infiltration are noted 2 mo after 25 mg DENA/liter was added to the water. H & E. × 600

plastic nodules. The cells forming the nodules stained less intensely with H & E than did the adjacent tissue and were PAS positive. Some cells in the nodules in treated fish were polymorphic and basophilic after the second month. Besides the proliferation of the hepatocytes, regeneration of the bile ducts was also observed; the epithelium was multilayered and dysplastic in the larger ducts. Sporadically, a large number of small bile ducts (visible in cross section) lay within the parenchyma, either singly or in groups. Sometimes, we observed cystically dilated structures lined by flattened cuboidal epithelium. A slightly increased amount of connective tissue surrounded these structures (fig. 3). In addition to the liver alterations, approximately 10–15% of the treated fish had developed hyperplasia and dysplasia of the esophageal and intestinal epithelia.

Neoplastic Alterations

Apparently, the DENA-induced liver cholangiomas that consisted of cystic structures lined by flattened, normal-appearing cells were benign. Cholangiocellular and hepatocellular carcinomas appeared in several areas (figs. 4A, 4B, 5) partly as nodules and partly as diffuse infiltrating masses. The cells comprising the nodules also infiltrated the surrounding liver tissue. Some tumorous nodules consisted of acidophilic cells, but others, mainly the most infiltrative ones, consisted of mostly basophilic tumor cells. Nodules consisting of both acidophilic and basophilic cells were found in a given liver. Metastases were not evident, but the continuous spread of tumors into the mesentery occurred occasionally. We identified early malignant tumors according to histological atypia and invasive behavior. Gastrointestinal tumors were only found in 2 DENA-treated fish: One was an esophageal papilloma and the other an intestinal polyp. Tumors were observed in various incidences in different groups (table 2). Even the nontumorous parts of the liver showed various alterations, such as degeneration, necrosis, hemorrhage, and hyperplasia, all variable within a given liver.

Effects of Treatment With N-N′*-Dinitrosopiperazine*

During the course of the experiments on the carcinogenic effect of *N,N′*-dinitrosopiperazine, a diffuse and nodular hyperplasia of the hepatocytes and cholangiofibrosis were observed in the livers of the guppies. The epithelium was hyperplastic and dysplastic in the gastrointestinal tract. In addition to these preneoplastic alterations, hepatocellular carcinomas in some fish (fig. 6), multiple papillomata in the esophagus in 1 fish (fig. 7), and an intestinal polyp in another were detected. The tumors found in the piperazine-treated fish were morphologically similar to those in fish treated with DENA. Nevertheless, not only were more tumors and precancerous alterations observed in the DENA-treated fish, but they appeared earlier.

Effect of Chemicals For Drug Use in Fish

Of the 10 chemicals intended to be introduced as antiarrhythmic, depressant, and antidepressant drugs in future use (table 1) that we administered to guppies, 2 (known by code number only) were determined to be potential carcinogens. The chemical coded KL-157 caused a diffuse hyperplasia of the hepatocytes and marked

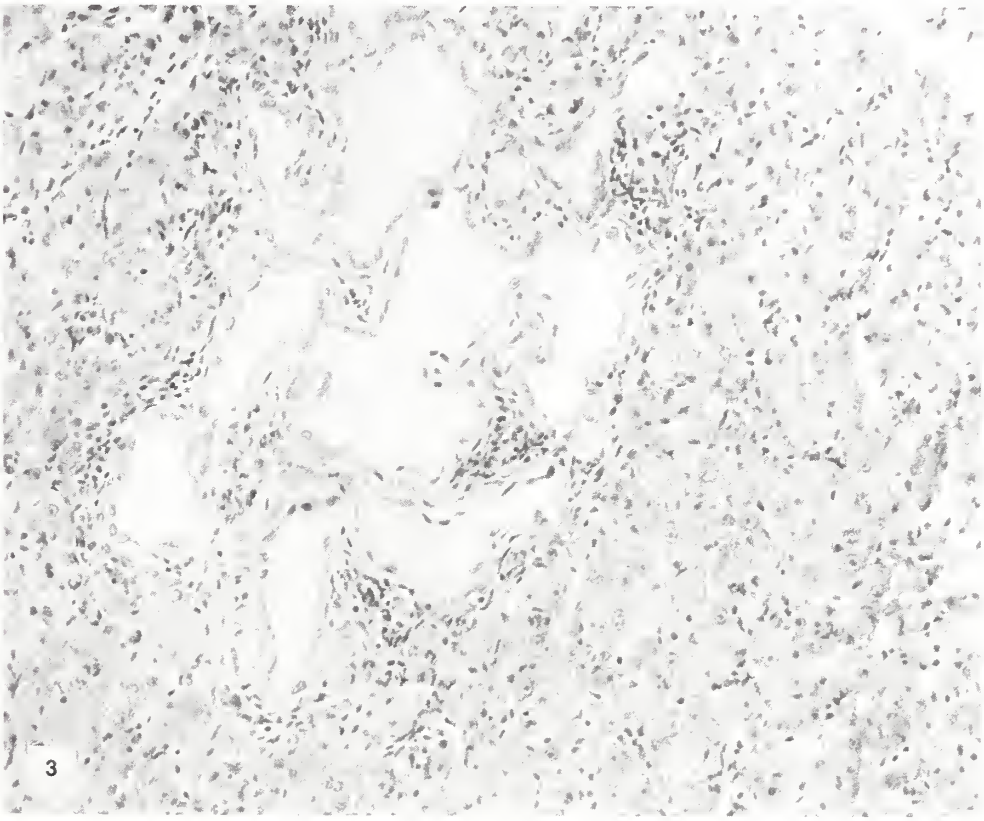


FIGURE 3.—Cholangiofibrosis and diffuse hyperplasia 2 mo after 50 mg DENA/liter. A slightly increased amount of connective tissue can be seen surrounding cystically dilated bile ducts which are lined with flattened epithelium. Proliferation of hepatocytes showing a slight polymorphism can be observed in the surrounding parenchyma. H & E. × 400

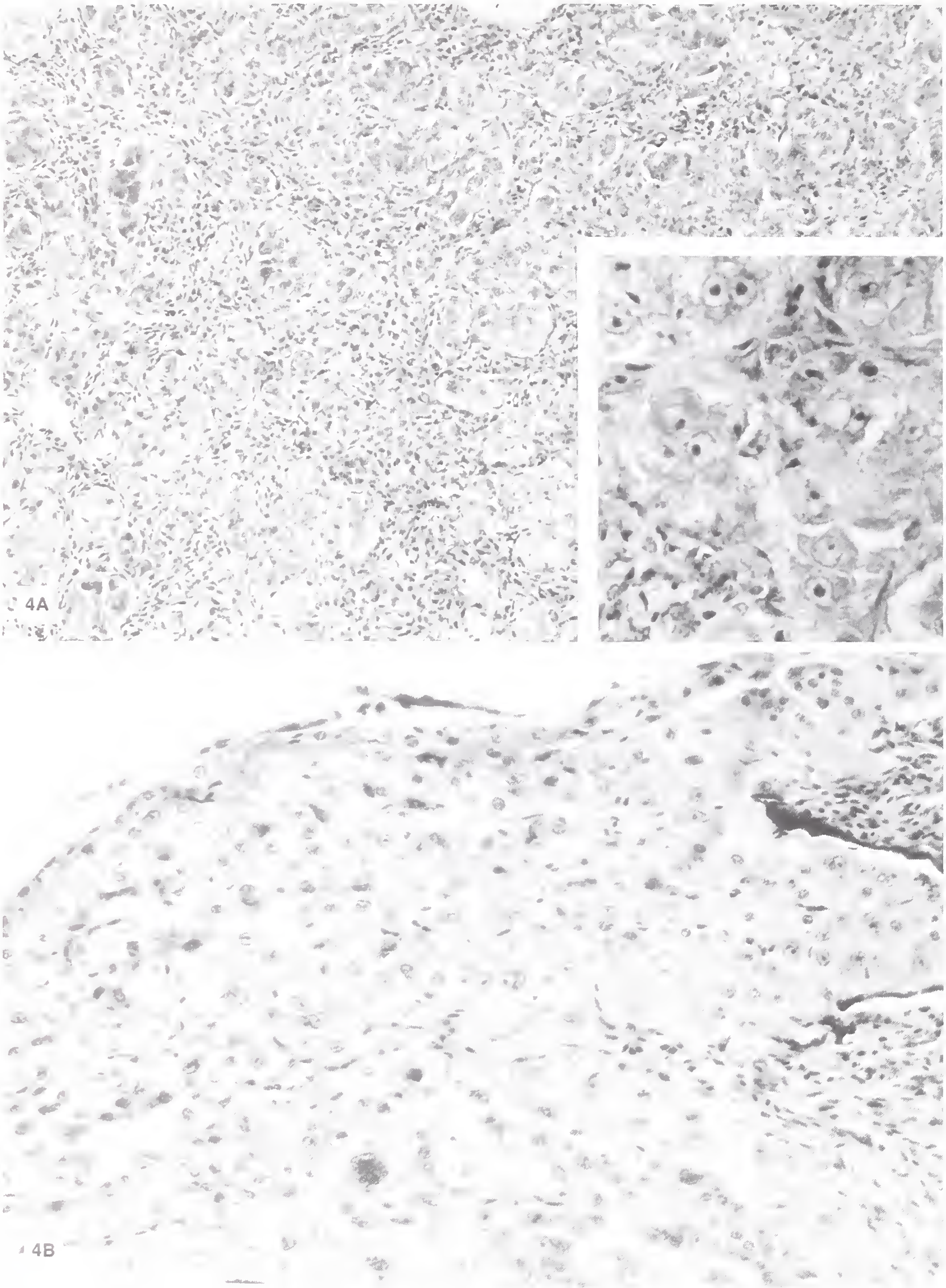


FIGURE 4.—Hepatocellular carcinoma A) 3 mo after 50 mg DENA/liter. *Note* nodular character of the tumor; cells are highly polymorphic (insert). H & E. × 250; insert × 1,000. B) Hepatocytes form primitive atypical trabeculae and show prominent polymorphism and infiltration of root of mesenterium (*right*) after 3 mo. H & E. × 400

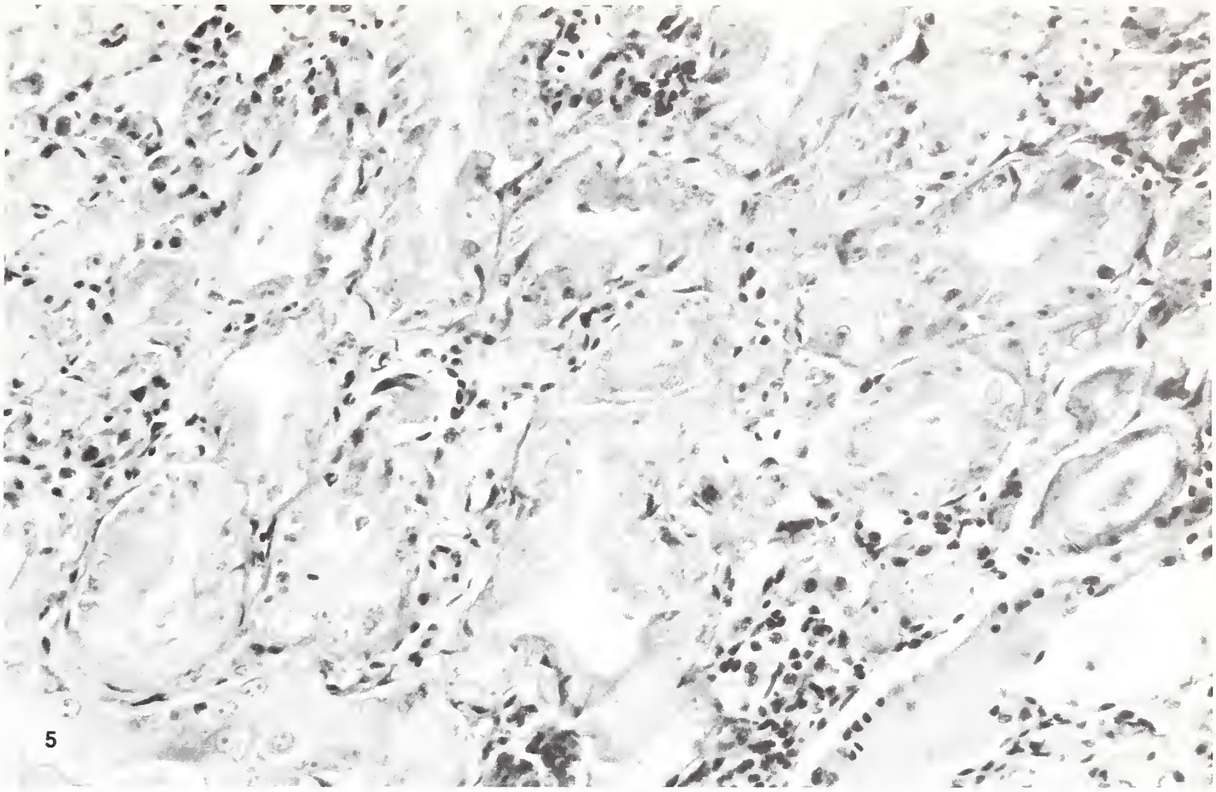


FIGURE 5.—Cholangiocellular carcinoma 3 mo after 50 mg DENA/liter. Atypia and infiltration of tumor cells are striking. H. & E. × 400

proliferation of small bile ducts by the end of the fourth month (fig. 8). These alterations were detectable in 20 of the treated animals (table 2).

In 25% of the livers of the fish treated with the compound coded KL-170, the diffuse and nodular hyperplasia of the hepatocytes as well as cholangiocarcinomas in 2 fish were detected by the end of the third month (fig. 9).

Table 2 presents the results of the screening experiments performed with the pharmaceutical chemicals from EGYT-Pharmacochemical Works and DENA-treated controls. The livers of the guppies exposed to the other compounds showed slight degenerative lesions; neither preneoplastic nor tumorous lesions could be observed.

DISCUSSION

Tumor research on aquarium fish has become increasingly significant in recent years since the discovery that fish could be superior indicators of the pollution of our water sources by carcinogenic substances (*16–19*). Of the several authors (*4, 6, 7, 20, 21*) who have written about the advantages of using aquarium fish in carcinogenesis studies, some consider fish to be suitable models for the screening of the possible carcinogenic effects of chemicals (*6, 21*). We would like to note the following advantages of using fish in screening for potential carcinogenic compounds:

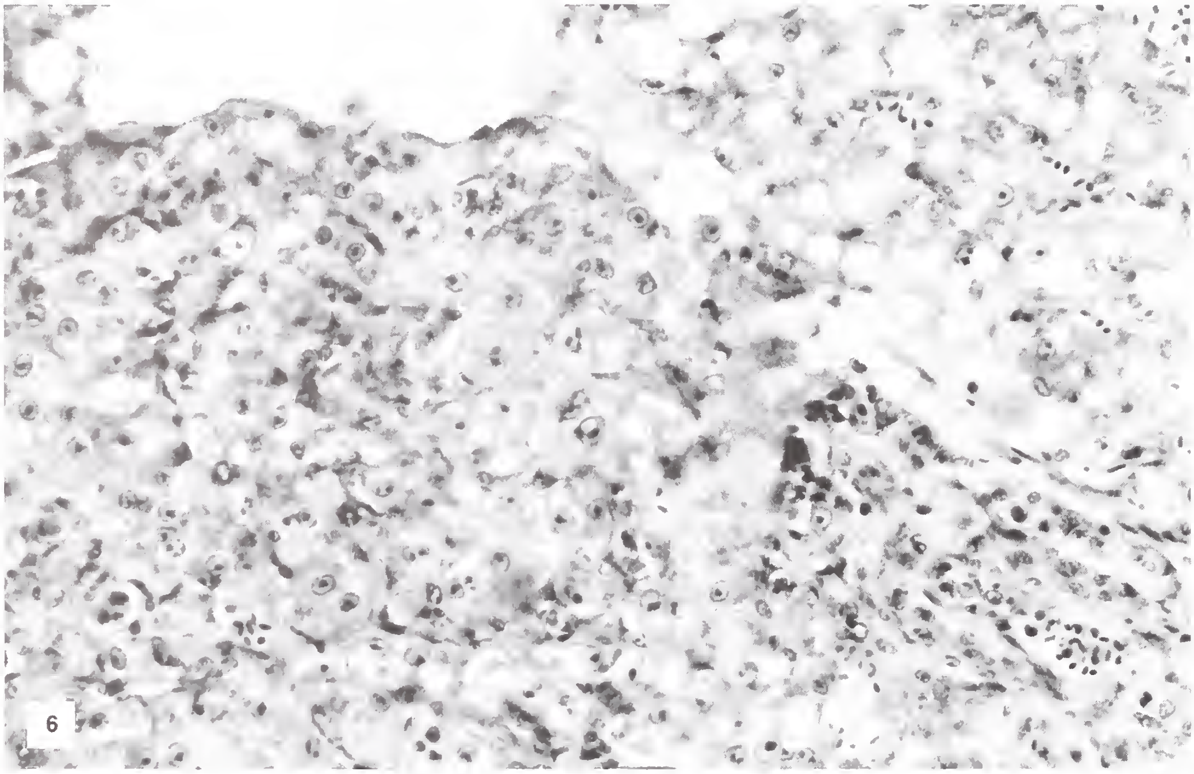
1) The experimental period is short when one screens substances which are water soluble or can be made water soluble by certain solvents.

2) The nature of fish enables three-way intensive contact of the animals with the chemicals under test simultaneously: the gill, integument, and gastrointestinal tract.

3) Fish experiments have proved to be cheaper than the traditional experiments on rodents.

The guppy is a frequently used test animal because it is readily accessible, hardy, and easily maintained (*4, 6, 9, 21, 22*). According to our studies, guppies have drug-metabolizing enzymes in their livers, although the aryl hydrocarbon hydroxylase activity is significantly lower than in rats. Our data agree with those of other authors (*23–26*), who described the presence of a microsomal enzyme system necessary for the ultimate transformation of proximate carcinogens in aquatic animals. However, this activity was lower in fish (*26*), which may explain the need for large doses of carcinogens if we are to achieve the tumor-inducing effect in fish (*27*). We did not determine the optimal conditions for assaying the enzyme activity of the guppy liver; therefore, alteration of the conditions (temperature, pH, etc.) may influence the actual aryl hydrocarbon hydroxylase activity.

In our studies, the guppy proved to be sensitive toward the carcinogenic effect of various chemicals. We consider it essential to mention that *N,N'*-dinitrosopiperazine, which is carcinogenic in rodents (*28, 29*), was also carcinogenic in fish. Under certain conditions, this compound may be formed from the piperazine component of the pesticide Triforine (*N,N'*-piperazinyl-bis-2,2,2,-trichloroethylidene-



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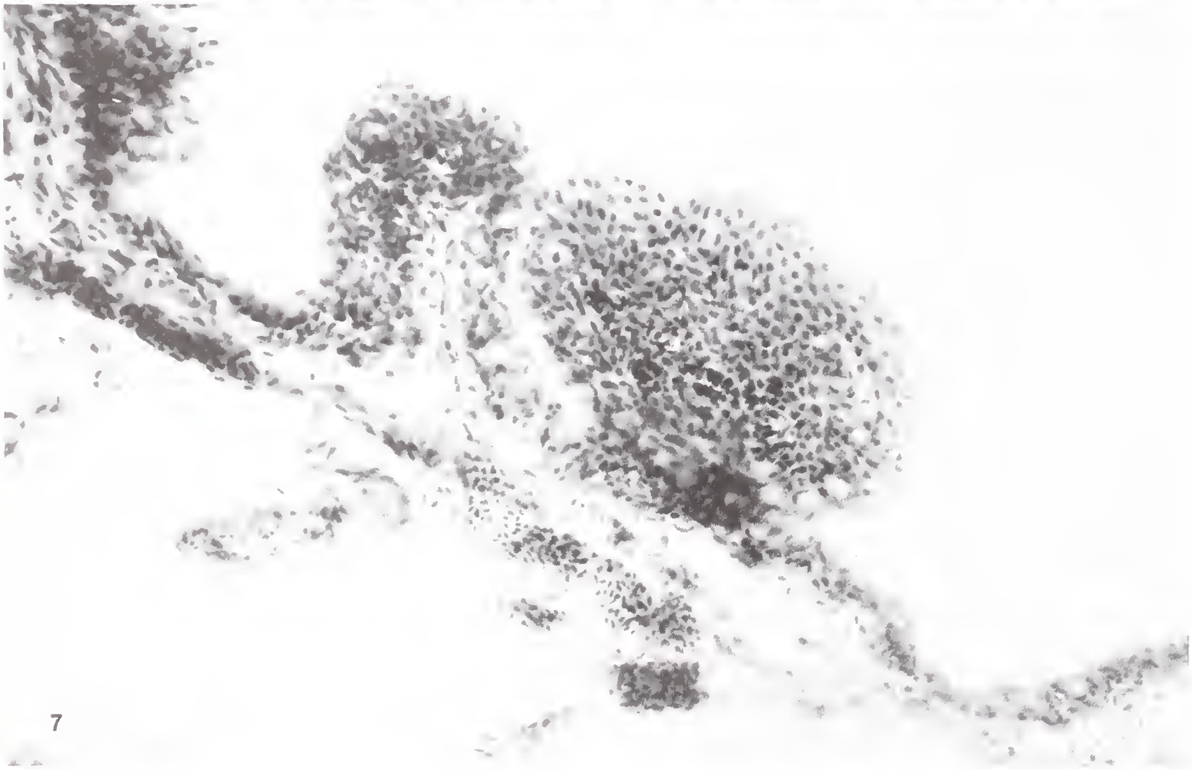

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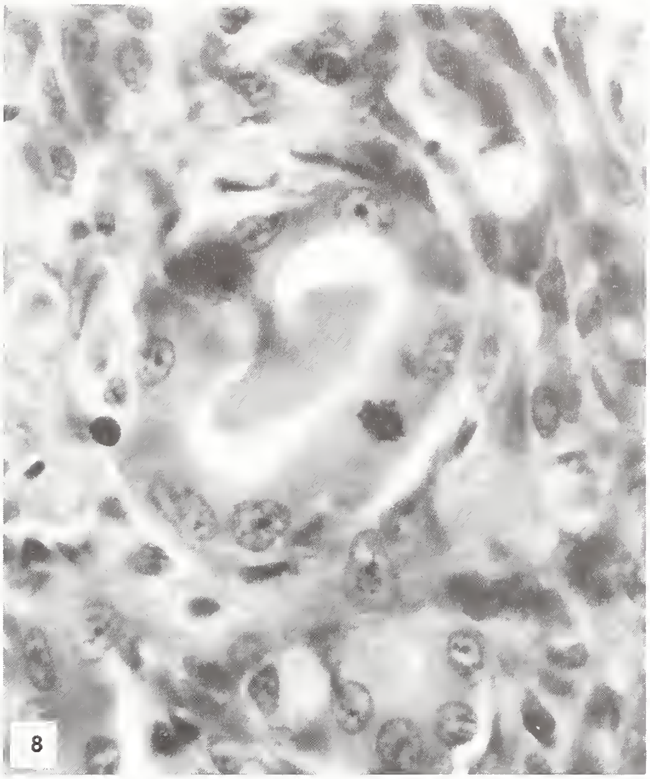
FIGURE 6.—Hepatocellular carcinoma resulting from treatment with *N,N'*-dinitrosopiperazine. Border between normal liver tissues showing fatty change (*upper right*) and the neoplastic lesions is clearly seen. Tumor is a solid mass of polymorphic cells. H & E. × 400

FIGURE 7.—Esophageal papilloma 1 mo after 100 mg *N,N'*-dinitrosopiperazine/liter. Increased numbers of epithelial cells are irregularly arranged but cellular atypia is not apparent. H & E. × 400



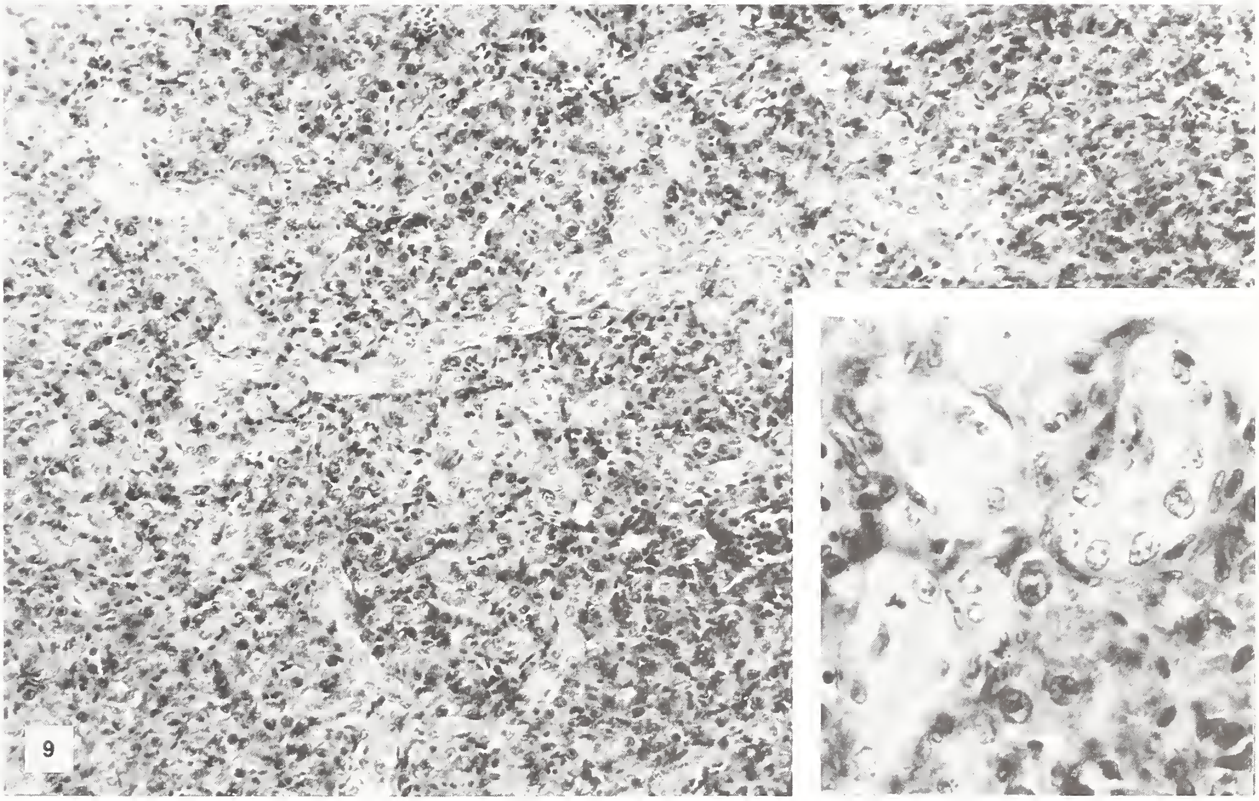


FIGURE 8.—Dividing cell in a proliferating bile duct 4 mo after 0.25 mg KL-157/liter. H & E. × 1,000

FIGURE 9.—Infiltrating cholangiocarcinoma in a guppy treated with 5 mg KL-170/liter after 3 mo. Cords of tumorous tissue are infiltrating the liver. Tumor cells form abortive canaliculi and show polymorphism. *See* insert. H & E. × 250; insert × 800

bis-formamide; Celeamerck, Ingelheim, Federal Republic of Germany) during decomposition (*30*).

The chemical coded KL-170, which caused cholangiocellular carcinoma in 2 of 20 animals, proved to be mutagenic by the Ames test and sister chromatid exchange studies by researchers in other laboratories.

Morphologically, certain liver alterations observed in our experiments were similar to those found in rodents or fish (*11, 31-33*). Some of these lesions were considered to be precancerous alterations. The presence of hyperplastic nodules showing slight or more pronounced polymorphism was described both in mammals (*34, 35*) and in fish (*6, 8, 9, 11, 31*); cholangiofibrosis was also observed in rodents (*36*) and fish (*6, 11*) during treatment with hepatocarcinogens. According to Bannasch and Massner (*36*), cholangiofibrosis represents an intermediate stage in the development of cholangiocellular tumors. At present, it is generally accepted that the hyperplastic nodules are the direct precursors of experimental hepatocellular carcinoma in rodents (*34, 37-39*). We wish to emphasize that, during our experiments, neither tumors nor precancerous alterations could be observed in the untreated fish. Therefore, the appearance of tumors, despite their low occurrence in the treated fish, should be considered as highly significant.

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Usefulness of *Cyprinodon variegatus* and *Fundulus grandis* in Carcinogenicity Testing: Advantages and Special Problems[1, 2]

Lee A. Courtney *and* John A. Couch[3]

ABSTRACT—*Cyprinodon variegatus* and *Fundulus grandis*, two species of cyprinodontid fishes studied extensively and used in toxicological and biological investigations, are compared as laboratory test animals. Their ecology, general biology, and suitability for various types of experimentation are examined. A laboratory system for exposure of these species at critical life stages (embryos, fry, juveniles) to suspect carcinogens is described. A discussion of the use, findings, and potential of these species in oncological studies and carcinogen assays is presented, particularly in regard to responses to 3 known or suspect carcinogens (trifluralin, benzidine, and aflatoxin). Finally, advantages and disadvantages or special problems in using the species as carcinogen test animals are reviewed. — Natl Cancer Inst Monogr 65: 83–96, 1984.

For years, the cyprinodontid fishes have been a source of abundant adaptable organisms for research. *Cyprinodon variegatus* Lacépède 1803, commonly known as the sheepshead minnow, an important species in the estuarine ecosystem, is widely distributed, easily collected, and well suited for laboratory culture and research. The general biology and ecology of *C. variegatus* have been studied extensively since 1900, and many references to systematics and collection date back well into the 1800s. During the past 10 to 15 years, the sheepshead minnow has been used routinely by scientists for testing toxicity of environmental pollutants and contaminants. Refined culture techniques have enabled researchers to hold and breed this adaptable species in flow-through, recirculating, and static systems year round at coastal facilities or in locations far from any salt water source. *C. variegatus* has proved valuable in acute and chronic toxicity tests, reproduction studies, tests for pathological and physiological responses, and, more recently, carcinogenicity.

Cyprinodontid fishes of the genus *Fundulus* have also provided researchers with excellent test organisms. *F. similis* Baird and Girard, *F. confluentus* Goode and Bean, *F. heteroclitus* Linnaeus, and *F. grandis* Baird and Girard have been used for various studies ranging from developmental investigations to pesticide testing to assay of lunar material collected during the Apollo Program (*1*). Extensive studies have been made of the life history, general biology, and ecology of many *Fundulus* species. For example, *F. heteroclitus* has been used widely in developmental and embryological studies for over 50 years (*2*) and much of what we know concerning fish embryology was learned with *Fundulus.*

Culture techniques have also been developed for *Fundulus* that allow the production of large numbers of fish for testing or commercial enterprise. The species of particular interest to us in the South Atlantic and Gulf Coast region is *F. grandis,* the Gulf killifish, a close relative of *F. heteroclitus.* Like *C. variegatus,* this species provides an available and easily cultured test organism. However, some significant developmental and physical differences do occur between *C. variegatus* and *F. grandis.* The latter species exhibits an extended embryological development, a longer maturation period, a larger size, and it offers a varied experimental application.

We present here a concise review and comparison of the general biology and ecology of *C. variegatus* and *F. grandis.* In addition, the suitability of each species as a laboratory test animal will be discussed, including culture and holding methods. A brief overview of the sensitivity of *F. grandis* and *C. variegatus* in toxicological testing will follow. Finally, we shall consider the use, findings, and potential of these species in oncological studies and carcinogen assays.

TAXONOMY AND SYSTEMATICS

The killifishes (order Cyprinodontiformes) are found throughout the world. The order was organized into 8 subfamilies under the family Cyprinodontidae by Myers (*3*); however, Rosen (*4*) raised 1 subfamily to familial rank and created a 2-family structure for the order. Taxonomic classification (to subfamily), according to Rosen's 2-family system, and general distribution of the Cyprinodontiformes, are shown in table 1. *F. grandis* and *C. variegatus* are both members of this order.

C. variegatus has occupied a stable taxonomic position since its original description by Lacépède in 1803 (*5*). However, *F. grandis* and several other *Fundulus* species have been the objects of some controversy regarding taxonomic position and relationships (*6–8*).

ABBREVIATIONS: ppt = parts per thousand; H & E = hematoxylin and eosin; LC50 = 50% mortality when the lethal concentration is administered.

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[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Supported by the Environmental Research Laboratory of the United States Environmental Protection Agency, Gulf Breeze, Florida 32561.

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TABLE 1.—*Taxonomic classification of the order Cyprinodontiformes to subfamily with general geographic distribution*

Family	Geographic area
Cyprinodontidae[a]	
Subfamily	
Cyprinodontinae	Mediterranean, Persian Gulf, Red Sea, Turkey, northwestern Indian Ocean, and portions of southern North America and northern Central America
Fundulinae	Spain, southern France, Turkey, portions of southern North America, Central America, and parts of the Caribbean
Lamprichthyinae[b]	Lake Tanganyika, East Africa
Orestiatinae[c]	Lake Poopo, Lake Titicaca area of South America
Pantanodontinae	East Africa and Madagascar
Procatopodinae	Africa
Rivulinae	Africa, India, Southeast Asia, East India, South America, Central America, and parts of North America and the Caribbean
Oryziatidae[d]	Japan, The Philippines, Celebes, India, Southeast Asia, and East Indies

[a] All but one genera of killifishes are included.
[b] Only a single genus is included: *Lamprichthys*.
[c] Only a single genus is included: *Orestias*.
[d] Only a single genus is included: *Oryzias*.

Similarities exist between certain members within the genus; for example, *F. grandis* Baird and Girard, *F. heteroclitus* Linnaeus, and *F. grandissimus* Hubbs represent a closely related group. Other trios and pairs of closely related species are also found within the genus (*8*). Whether these represent true species, variants, or subspecies has been debated, and various methods of grouping have been used (*7–9*) with conflicting results, leaving specific distinctions within these groups in question. The significance of these questions is uncertain, and until research shows otherwise, the bearing these minor species or sibling species differences may have on species selection for carcinogen testing may be considered of minor importance.

DISTRIBUTION AND ZOOGEOGRAPHY

Both *C. variegatus* and *F. grandis* are readily available for experimental use. *C. variegatus* ranges southward from Cape Cod, Massachusetts, around the peninsula of Florida, along the Gulf of Mexico and into northern South America [fig. 1; (*10–12*)]. *F. grandis* occupies a more limited territory (fig. 1), occurring from the Matanzas River in northeastern Florida down to the Florida Keys and extending northward again around the Gulf of Mexico and down into Mexico (*13, 14*). Interestingly, *F. heteroclitus*, previously noted as a close taxonomic relative of *F. grandis*, has a range extending northward on the east coast from the northern range limit of *F. grandis* to Anticosti Island in the Gulf of St. Lawrence (*13, 14*). Their distributions thereby provide 2 closely related *Fundulus* species and *C. variegatus* as available test animals along the entire eastern and Gulf coasts of the United States and portions of Central and South America.

The habitats occupied by these species are overlapping, and seine collections within adjoining ranges occasionally result in the simultaneous capture of both. *C. variegatus* prefers shallow brackish areas of pools (small, sandy-bottomed sites in bays, coves, bayous, creeks, tidal cuts, or channels) that tend to be drained or left isolated during low tides (*15–18*). Kilby (*15*) observed that fish select areas where little or no submerged aquatics are present, locations offering access to adjacent marshes for food and shelter from the shallows of open bays and large bodies of water. In addition, habitat preference is influenced by fish size. Only adult or subadult fish are collected in water courses; fish of all sizes are taken from pools, coves, and marshes (*15*).

The *F. grandis* inhabits similar habitats in the estuarine system. Kilby (*15*) stated "species which are characteristic of the water-courses include *F. g. grandis* and *F. similis* when taken with *C. v. variegatus* . . . ." He further states that "*F. g. grandis* occurs most abundantly in outer pools, water-courses, and, to a lesser extent, open waters. . . ." Data gathered by Christmas (*19*) and McIlwain (*20*), and field collections conducted by staff at the Gulf Breeze, Florida, Environmental Protection Agency Laboratory (unpublished observations) support these findings.

Both species are euryhaline. *C. variegatus* has been collected in waters ranging from 0 (*15*) to 142.2 ppt (*21*).

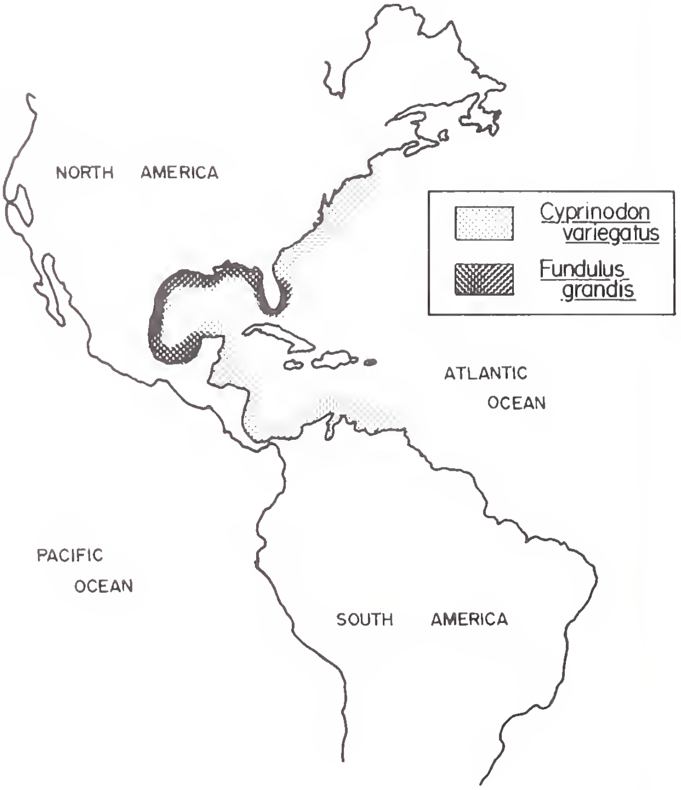


FIGURE 1.—Geographic distribution of *C. variegatus* and *F. grandis*.

Simpson and Gunter (*21*) indicated that high salinity is not a typical or preferred situation but occurs when fish are trapped in ponds where evaporation results in increased salinity. Normal salinity ranges for *C. variegatus* are from 0 to 35.6 ppt, with 20 ppt or less preferred (*15, 22*). *F. grandis* favors salinities of 2.0 to 22.5 ppt (*15, 19*), but, like *C. variegatus*, sometimes occurs at salinity extremes of 0.05 to 76.1 ppt (*21, 23*). An extensive review of salinity tolerances for *F. grandis, F. heteroclitus*, and other *Fundulus* species was done by Griffith (*24*).

C. variegatus and *F. grandis* are also eurythermal. Collections and investigations of *C. variegatus* by Warlen (*17*), Forman (*22*), Copeland (*25*), and Westman and Compton (*26*) indicate a temperature tolerance of 2° to 39° C. Christmas (*19*) reported gathering *F. grandis* at temperatures ranging from 5.0 to 34.9° C. The tolerance of salinity and temperature extremes exhibited by both species indicates that *C. variegatus* and *F. grandis*, hardy species in nature, would be adaptable as test animals in the laboratory.

Moreover, a point of further interest is the transportability of these species, especially that of *C. variegatus*. Embryos, fry, juveniles, or adults can all be transported several hundred kilometers in plastic bags if provided periodic oxygenation. This enables researchers at inland laboratories or locations outside the species' normal ranges to utilize them. From 1977 to 1981, several thousand *C. variegatus* of various life stages were transported from the Environmental Protection Agency Laboratory in Gulf Breeze to an inland university with excellent results.

MORPHOLOGY AND LIFE CYCLE

Sexual dimorphism in adult *C. variegatus* is marked by differences in coloration. In general, the body of males is olivaceous and marked with vertical, dark stripes along its sides. During breeding, the anterior dorsal area from the head to above the pectoral fins turns a deep, iridescent steel blue. The most obvious male coloring characteristic, even in nonbreeding males, is a jet-black bar at the tip of the caudal fin. The sex of young males and fish which, through captivity and manipulation have lost their breeding colors, is easily distinguished by this mark. Females are far less colorful and can be distinguished by a dark spot near the posterior base of the dorsal fin.

Sexual dimorphism in *F. grandis* is also striking. Sex determination is easiest during breeding; at this time, males are recognized by pearly white spots located on the sides and fins. Generally, the body is dark green with highlights of yellow and orange on fins and belly. Females are generally plain and have an olive and silvery body with faint silvery vertical bars. No white spots are found on the body or fins.

The life cycles of *C. variegatus* and *F. grandis* are depicted in figure 2, with approximately comparable developmental stages and average developmental times. Considerable work has been done on the embryology of *C. variegatus* (*27*) and *F. heteroclitus* (*28*). Our observations seem to indicate that the life cycle of *F. grandis* is similar to that of *F. heteroclitus*. Published work on the latter type can serve as an excellent guide in the study of either *Fundulus* species.

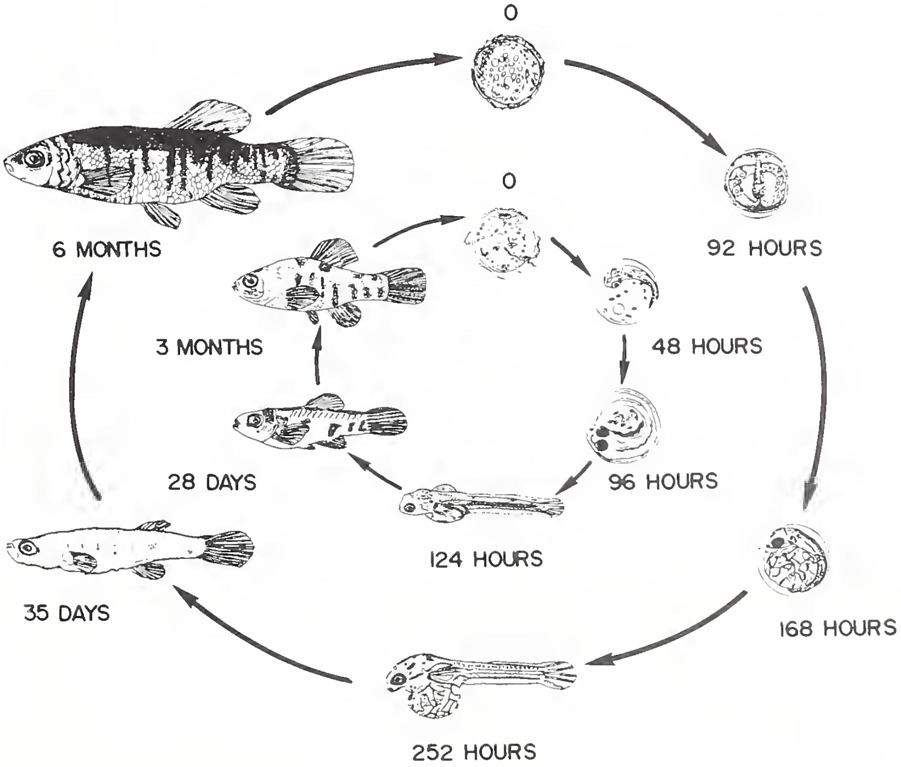


FIGURE 2.—Life cycles of *C. variegatus* (*inner circle*) and *F. grandis* (*outer circle*). Approximately comparable life stages are depicted with average developmental times. Stages are representative of those critical to carcinogen test regimens.

The rapid development of *C. variegatus* makes it an excellent test organism. Hatching, dependent on incubation temperature, occurs in approximately 5 to 15 days, usually 5 to 7 days, unless the temperature drops below 25° C. Generation time under laboratory conditions has been reported at about 3 months (*29*).

F. grandis develops more slowly than *C. variegatus*. The reported developmental period to hatching is 14 to 28 days and depends on the incubation temperature (*30, 31*); we observed similar periods at our laboratory. Tatum et al. (*31*) reported that growth to approximately 56 mm occurred within 52 days at large "grow-out" ponds. Observations on growth of the progeny of feral *F. grandis* spawned in our laboratory have shown reduced growth in comparison with rates reported in large pond operations. Likely causes of this decreased growth are 1) restricted housing space in the glass aquaria used, and 2) a dietary consideration which will be discussed later. These progeny, healthy in appearance and sexually differentiated, have been raised in small 60-liter aquaria for over 20 months but have failed to spawn.

Both *C. variegatus* and *F. grandis* are morphologically typical teleosts. Major organs and tissues in these species generally do not depart in their location or gross form from comparable features in most typical teleosts. In our experiences with *C. variegatus*, only a few exceptions have been noted, the most notable is the possible absence of Stannius corpuscles. Though many sectioned specimens have been examined, we have been unable to identify histologically these structures in anatomic regions where reported in other species, or in any other region of the fish. However, we may have either overlooked these tissues or they are uniquely formed and located in *C. variegatus*. The normal liver histology of this species is variable between specimens and depends on several factors, such as nutritional history and season. Hepatocytes vary in degree of vacuolation caused by removal of glycogen and fat during routine slide preparation. Livers of well-fed specimens (fig. 3) show a great degree of vacuolation (e.g., glycogen and lipid reserve), whereas those of starved specimens show little or no vacuolation (fig. 4). Acini of exocrine pancreas surrounding branches of the hepatic portal vein (fig. 4) and hepatic bile ducts are occasionally found in liver sections.

HISTOLOGICAL PROCESSING

Whole specimens, from zygotes to small adults, can be processed histologically with little difficulty. Figure 5 shows a parasagittal section of a 96-hour *C. variegatus* embryo. Developing organs and organ systems can be studied easily when serial sections are made. Liver differentiation has already occurred at this stage of development, i.e., 96 hours at 26° C (fig. 6); note the structure of hepatocytes in comparison with adult liver sections. Larger specimens (fig. 7) require decalcification before dehydration and embedding. Whole specimens up to 35 mm standard length can be processed and then cut on a longitudinal horizontal plane before embedding. Both halves of the fish are then embedded in the same paraffin block with the cut face on the sectioning plane. Using this method, a researcher can study all organs and major skeletal features with a few selected serial sections from various planes.

At present, the detailed histology of *F. grandis* is under investigation but generally appears similar to that of other teleosts. Embryos and young fish can be processed and studied by the methods described for *Cyprinodon*.

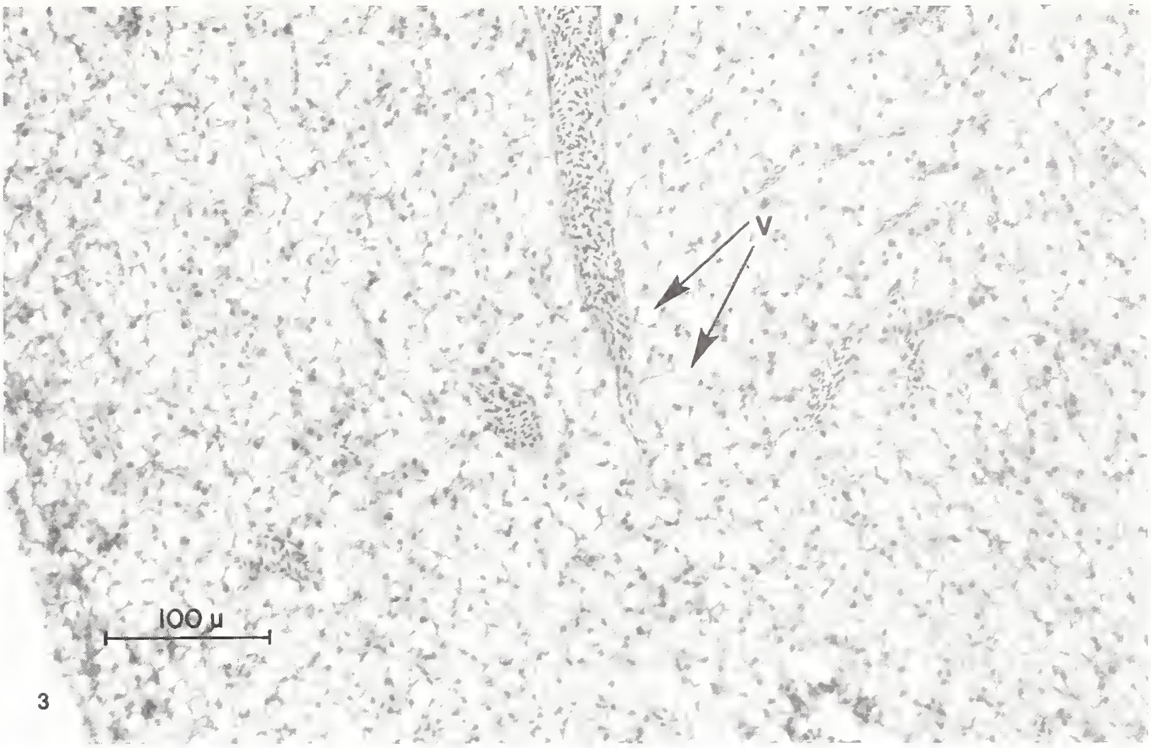
CULTURE

C. variegatus is an easily handled and maintained laboratory research animal. Culture techniques have been developed with either closed systems, under-substrate filter aquaria, or flow-through marine systems (*29, 32*). Adaptations of these laboratory techniques have also been applied successfully to spawning of feral *F. grandis* (unpublished observation), and methods for large-scale commercial production have been developed (*20, 31, 33*) in attempts toward maintenance of the supply of *Fundulus* to the bait industry. Additional culture information for *F. heteroclitus* (e.g., *34, 35*), which is applicable to *F. grandis* as well as other species, is available.

Basically, laboratory culture of *C. variegatus* consists of acclimation (to temperatures between 26° and 30° C and a 12-hour light/dark photoperiod) and conditioning, i.e., feeding ad libitum (*32*). Laboratory breeding can be performed year round once these conditions are met; approximately 2–4 weeks is required for acclimation and preparation of the specimens (*32*).

Once gravid fish are available, 1 of 2 basic methods of culture can be followed, depending upon several factors, including numbers of breeders available, expendability of breeders, and numbers of eggs required for testing. Natural spawning methods (*32*) may be used if parent fish cannot be killed, or if a limited number of eggs are required. With this method, several females are housed with 1 or 2 males in a breeding chamber consisting of a glass enclosure with a large mesh nitex screen bottom (*36*). Natural spawning occurs and the fertilized eggs sink through the mesh bottom. These are collected on a removable screen tray fitted below the enclosure. When 4 to 5 spawning females are introduced to the spawning chamber in the afternoon, between 50 to 120 embryos can be harvested the next morning.

However, if fish are plentiful or expendable, or, most importantly, if large numbers of zygotes are required with critical control over fertilization time, manual spawning methods are indicated (*32*). In this method, acclimated adult females are either dissected or stripped of mature ova which are then placed in a small quantity of seawater. A large harvest of viable ova is ensured by administration of 2 ip injections of human chorionic gonadotropin on consecutive days, beginning 3 days before the fish are killed. The hormone acts as a hydrating agent, inducing maturation of the ova present; this treatment is optional, and the entire manual spawning procedure can be used without injections. Once the mature eggs are collected, the testes of several males are removed and macerated, and milt is introduced into the seawater containing the ova. After approximately 1 hour, the fertilized zygotes can be removed and manipulated as desired.



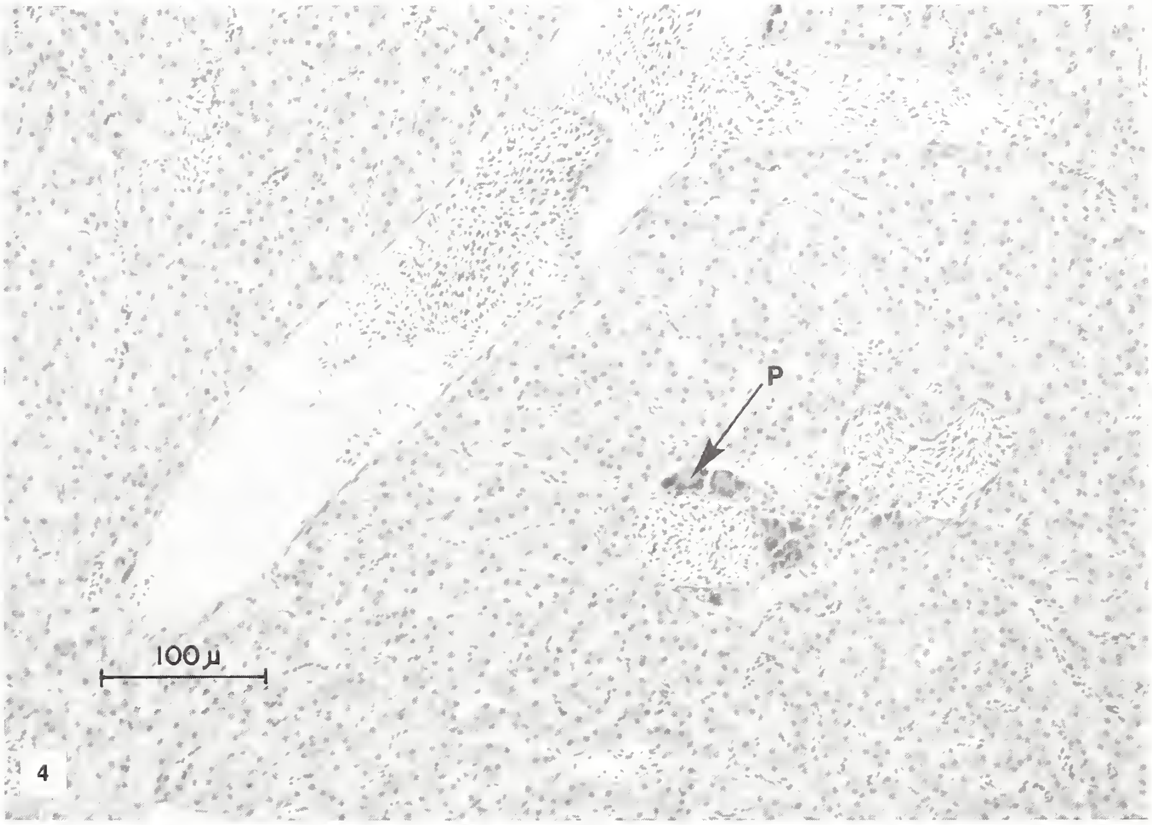
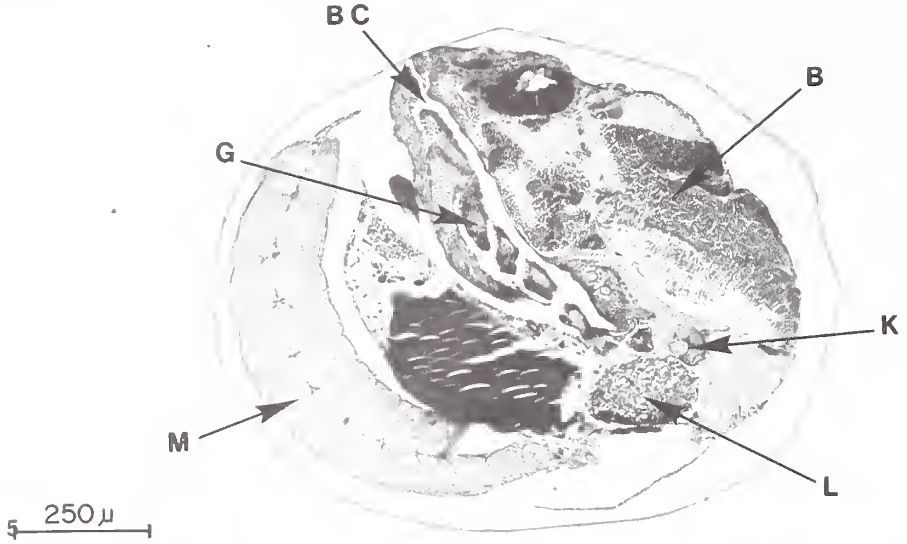


FIGURE 3.—Section of liver from well-fed *C. variegatus*. Hepatocytes show a great degree of vacuolation caused by extraction of stored lipid and glycogen from cells during histological processing. Distribution of lipid reserves is represented by discrete smooth edged vacuoles (V) dispersed throughout the tissue. H & E. × 225

FIGURE 4.—Liver section of a starved individual. Little or no vacuolation is present and hepatocytes appear dense. Acinus of exocrine pancreas (P) is associated with the hepatic portal vein. H & E. × 225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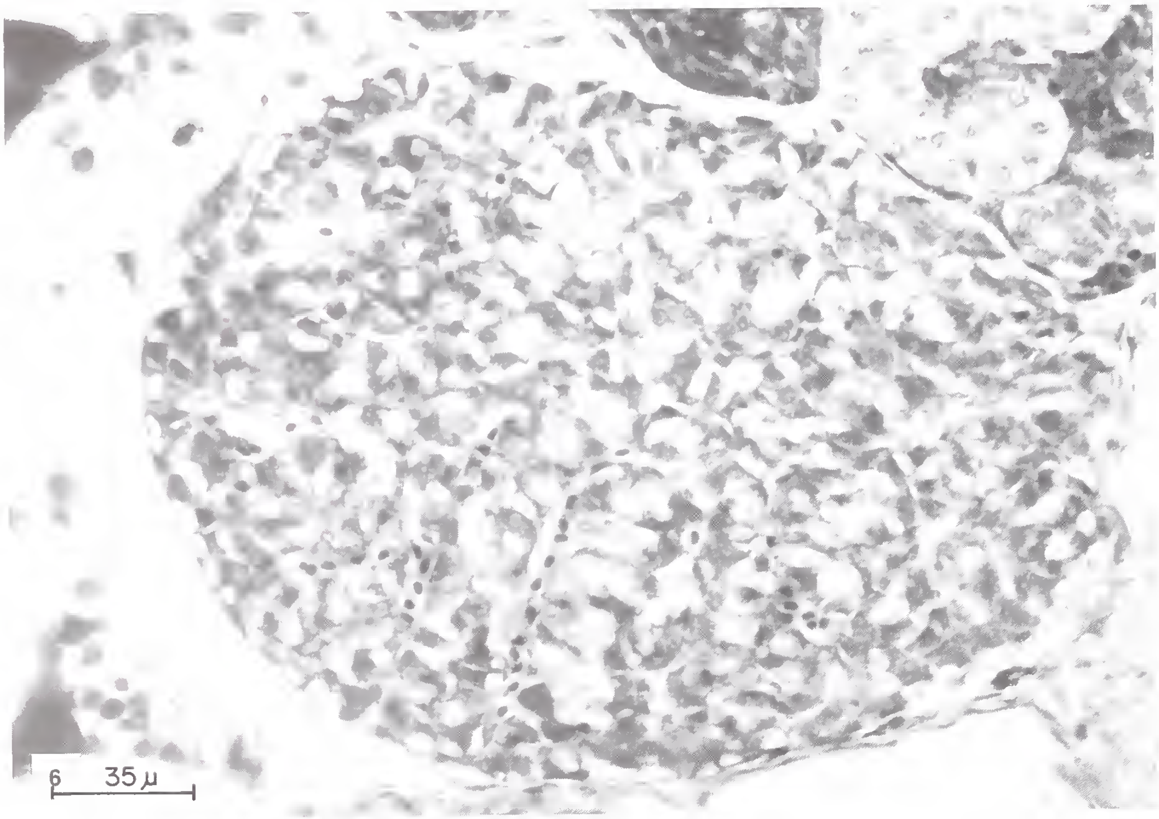

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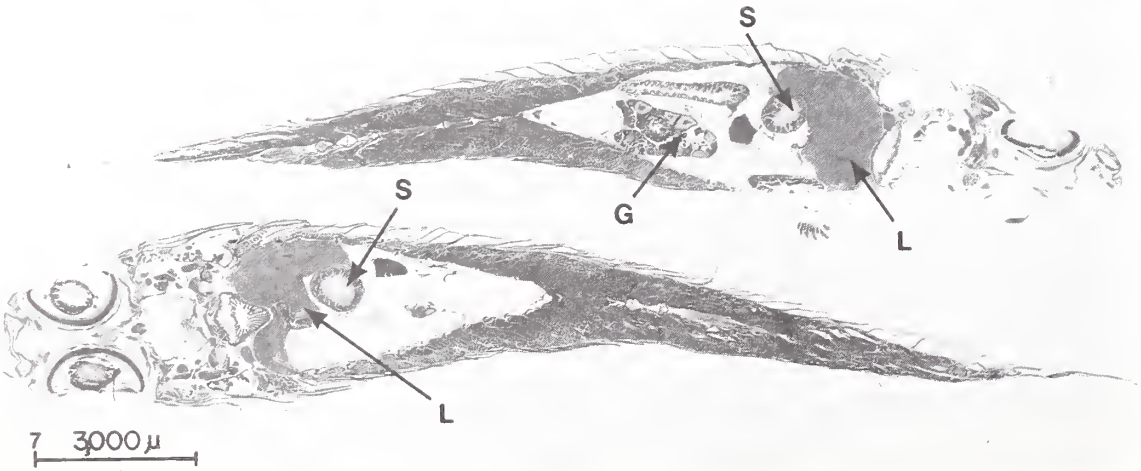
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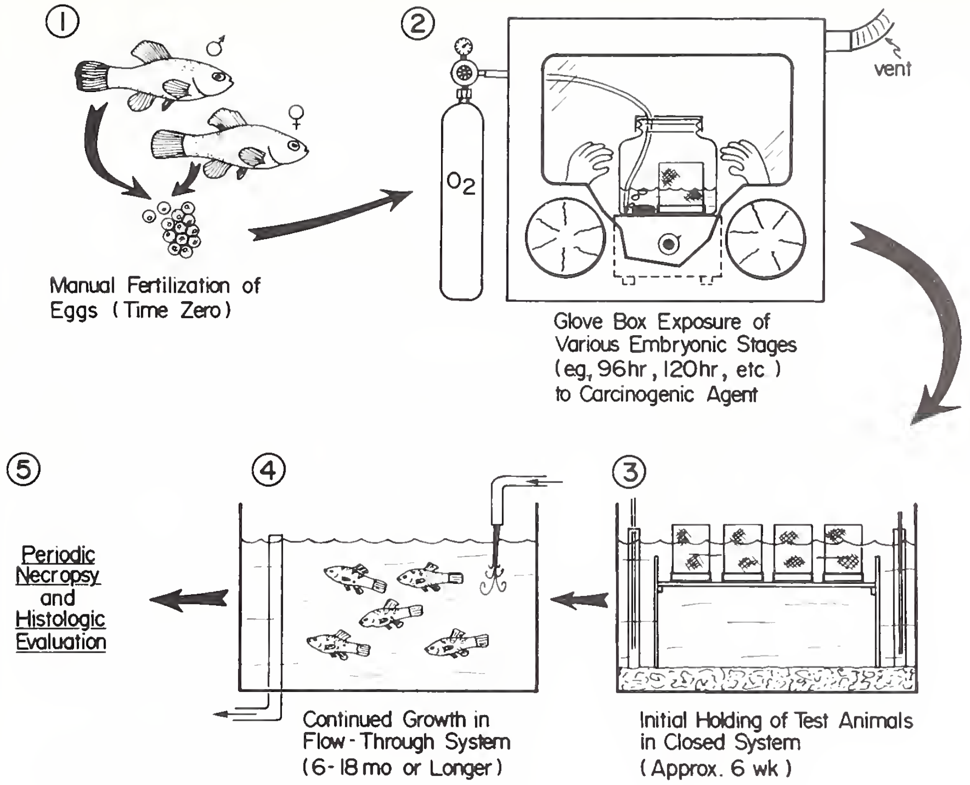
①
♂
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Manual Fertilization of Eggs (Time Zero)
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O_2
Glove Box Exposure of Various Embryonic Stages (eg, 96hr, 120hr, etc) to Carcinogenic Agent
⑤
Periodic Necropsy and Histologic Evaluation
④
Continued Growth in Flow-Through System (6-18 mo or Longer)
③
Initial Holding of Test Animals in Closed System (Approx. 6 wk)

FIGURE 8.—Test scheme for static pulse exposure of selected life stages (e.g., embryos, larvae) to suspect or overt carcinogenic agents. 1) Desired test organisms are obtained by manual methods. 2) Exposure for desired duration of selected stages is performed in closed containers within glove box. Test organisms are held in egg cups within the exposure chamber; a stirring bar evenly distributes and circulates the test agent through the egg cups. Periodic oxygenation of water prevents depletion of dissolved oxygen during exposure. 3) After initial decontamination of test animals in glove box, additional depuration can be accomplished in recirculating systems. 4) This action is followed by extended holding under flow-through conditions, or test animals can be transferred directly to flow-through holding facilities. 5) Multiple observational end points possible during the holding period include physiological, behavioral, biochemical, and histological measurements and assessments.

If stock-rearing is the goal, several hundred embryos can be held in 2,000-ml aerated separatory funnels for a week or more after hatching, then transferred to larger aquaria for grow-out. For testing purposes, 20 to 25 embryos can be held in egg cups, constructed of petri dish bottoms and nitex screen placed in trays designed to provide a fluctuating water level, with excellent results (*37, 38*).

Culture methods described above have been applied successfully to field-collected *F. grandis* (unpublished observation). Although we have held these cultured fish for over 20 months, to date we have had no spawning activity from these specimens.

TEST SYSTEMS

Numerous assay systems have been used in the testing of aquatic organisms. Choice of assay system depends on many factors ranging from type of compound tested to goals of the research project to personal preferences of the researcher.

In our laboratory, we used 2 basic systems in the assay of carcinogens on marine fishes. The first was long-term continuous exposure of various life stages of *C. variegatus* to the herbicide and suspect carcinogen trifluralin according to a system detailed in (*38*). This system, which consists of a water-toxicant delivery system and exposure tanks with various life-stage holding capacity, was for continuous exposure; however, it also could be used for pulse or one-time exposures. We had good results with a simple toxicant mixing chamber; toxicant concentrations ranged between 1 and 5 μg/liter during a 19-month period.

By another assay system (fig. 8), we exposed under static conditions embryos at various developmental stages (critical life stages) to a short single dose of the overt carcinogenic agent aflatoxin B_1. Exposures were conducted in a vented glove box in covered glass containers with the embryos held in egg cups. After exposure, the embryos were rinsed and transferred to uncontaminated egg cups before removal from the glove box, which greatly reduced possible transfer of the carcinogenic agent to the outside

FIGURE 5.—Parasagittal section of a 96-hour embryo of *C. variegatus*. Developing liver (L) can be seen as well as many other organs: brain (B), kidney (K), gill arches (G), buccal cavity (BC), caudal musculature (M). H & E. × 75

FIGURE 6.—Higher magnification of liver from 96-hour embryo of *C. variegatus* in figure 5. H & E. × 570

FIGURE 7.—Longitudinal horizontal sections of adult *C. variegatus*. Specimen was decalcified before processing, and entire fish was cut in the longitudinal horizontal plane immediately before embedding. Both halves of fish were embedded in the same paraffin block with the cut face on the sectioning plane. Therefore, sections pictured represent two planes of the same specimen. All organs and major skeletal features can be studied with this method with selected serial sections from various planes. Sections of liver (L), stomach (S), gonads (G) and other tissues are represented. H & E. × 7.4

environment. After transfer from the exposure cup and several rinses, most of the unabsorbed and unadsorbed compounds were removed; thus the health hazard was reduced. Once exposure and decontamination were completed, embryos were maintained in preconditioned recirculating aquaria through hatching and for several weeks thereafter. At that time, the young fish were moved to larger flow-through holding aquaria. This system, patterned after methods described by Hendricks et al. (*39*) for use with rainbow trout embryos, provided a safe, inexpensive method of one-time exposures of embryos and larval fishes. Exposures with *C. variegatus* were executed with ease and with deaths of fewer than 10% of the fish during the first 6 weeks.

NUTRITION

Nutritional requirements for *C. variegatus* and *F. grandis* differ. Fry of both species thrive on newly hatched brine shrimp (*Artemia* sp.). Young *C. variegatus* should have their diet supplemented with flake food (e.g., BiOrell or Tetramin)[4] and, as their size increases, with frozen adult brine shrimp which has been thoroughly thawed. Adults can be maintained with excellent results on a diet of equal parts flake food and frozen brine shrimp (*40*). We followed a similar feeding regimen with *F. grandis,* and although the fish appeared healthy at sexual differentiation, some growth retardation was noted. Therefore, young adult *F. grandis* should be fed, or, at least a portion of their diet (~50% or more) should be a commercial floating trout feed, such as Purina Trout Chow[4] (*31*), possibly supplemented with vitamins (*20*). This diet has shown promise in studies with pond-reared fish reported by McIlwain (*20*) and Tatum (*31*). All dietary ingredients should be analyzed for possible contaminants, such as pesticide residues or heavy metals. Commercially prepared trout food should be screened for aflatoxin contamination, due to the use of cottonseed oil and other ingredients in its preparation.

ROLES IN TOXICOLOGICAL TESTING

Toxicological testing with *C. variegatus,* begun in the last 30 years, has been extensive, and acute toxicity studies proliferated in the last two decades. Chronic toxicity studies were pioneered in 1974 at the Gulf Breeze Laboratory. Numerous compounds, including polynuclear aromatic hydrocarbons, insecticides, herbicides, and others, have been tested against this species, which displayed its responsiveness and sensitivity as a test organism. A detailed review of the toxicological uses and results of *C. variegatus* would be impractical and unnecessary here, but a few examples illustrate the responsiveness of the species.

Acute toxicity tests have shown that the sheepshead minnow's sensitivity to toxicants is comparable to that of other fishes from similar ecosystems. Schimmel and Hansen (*29*) reported comparisons of 48-hour effective concentrations for the teleosts *C. variegatus, F. similis, Leiostomus xanthurus,* and *Mugil cephalus* exposed to endrin, dieldrin, and dichlorodiphenyltrichloroethane, which indicated comparable susceptibility. Ninety-six-hour exposures of *C. variegatus* to endrin (LC50 = 0.34–0.37 μg/liter) showed comparable susceptibility with data generated on 10 species of estuarine fish [*C. variegatus* was more sensitive than 6 of these species (*36*)]. Furthermore, comparative toxicological responses were observed for *C. variegatus* and *Lagodon rhomboides* in tests with hepatochlor (*41*); these species, however, were less sensitive than was *L. xanthurus.* In other instances, the sensitivity of the sheepshead minnow was less than that of other estuarine species; e.g., in acute studies it was approximately ten times less sensitive to kepone than was *L. xanthurus* (*42*).

In chronic, life-cycle toxicity studies, *C. variegatus* has also shown its responsiveness and usefulness. For example, with kepone, detrimental effects were observed in *C. variegatus* at concentrations a thousandfold less that of previously reported 96-hour LC50 values (*43*). Results of chronic endrin toxicity tests, on the other hand, corroborated acute data for the chronic effects level of 0.31 μg/liter and acute 96-hour LC50 concentrations of 0.34–0.37 μg/liter (*36*). These data and others suggest that *C. variegatus* is an indicative and sensitive estuarine species.

The use of *F. grandis* as a toxicological test organism has not been as extensive as that of *C. variegatus;* however, several studies indicate that it may be a responsive, useful estuarine species. For example, Ernst and co-workers (*30*) noted pathological effects in embryos exposed to 25% concentrations of the water-soluble fraction of No. 2 fuel oil, a concentration unlikely to be found in the aqueous environment but potentially available in petroleum-laden sediments into which developing embryos may settle. Also, *F. grandis* has been used in investigations of toxicant effects on fin regeneration (*44*), of hexachlorobenzene and hexachlorobutadiene [20-hr LD50: 1,700 μg/kg body wt observed for the latter chemical (*45*)], and numerous studies involving the effects of salinity, temperature, photoperiod, and other parameters on a variety of serum constituents (*46–50*). Although these latter citations do not involve toxicological testing, they do illustrate an important consideration in the use of *F. grandis* in carcinogen assay when compared with smaller species such as *C. variegatus,* i.e., that of the adaptability of *F. grandis* to serological studies. The adult *C. variegatus* averages 48 mm standard length for males and 45 mm for females and is not an optimal test animal for blood serum studies. Pooled sample methods have been performed at our laboratory with some difficulty and with fairly large numbers of fish required (*51*). The small size of *Cyprinodon* and low volume of collectable blood make individual samples impractical. *F. grandis,* however, the standard length of which can range to 100 mm and greater, is large enough to permit blood chemistry studies of individuals, some of which are currently under consideration.

PREVALENCE OF IDIOPATHIC TUMORS IN *CYPRINODON* AND *FUNDULUS*

Examination of hundreds of specimens of *C. variegatus* from both feral and laboratory populations provides little evidence that the sheepshead minnow has a high natural

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[4] Use of trade names in no way indicates endorsement by the U.S. Environmental Protection Agency.

TABLE 2.—*Idiopathic tumors reported in* F. heteroclitus, F. grandis, *and* C. variegatus[a]

Species	Tumor type	Reference
C. variegatus	Thyroid (adenoma)	(*52*)
	" "	(*53*)
	Odontogenic (ameloblastoma)	(*54*)
F. heteroclitus	Thyroid (adenoma)	(*52*)
	Integumentary (fibroma)	Yevich PP: Submitted for publication
	"	RTLA Spec. No. S0000204
	" (fibroepithelioma)	Paperna I: Submitted for publication
		RTLA Spec. No. S0000762
F. grandis	Possible stem cell (probable chromatophoroma)	Couch JA: Unpublished observation

[a] Data are from the Smithsonian Institution: Registry of Tumors in Lower Animals.

tumor prevalence. We have yet to find an invasive idiopathic neoplasm in *Cyprinodon*. A possible explanation for this low incidence is that fish, burdened with neoplasia, would be subject to a higher degree of predation than normal (due to decreased mobility, disorientation, etc.) and, therefore, would not be collected. The literature (table 2) also suggest that neoplastic development is rare in this species. Nigrelli (*52*) reported thyroid tumors in *C. variegatus* from the New York Aquarium that were characterized by increased follicular and afollicular elements. The tumors were classified as interacinous adenomas. Another aquarium-held sheepshead minnow with a similar tumor was reported by Lightner and Meineke (*53*); however, their tumor was classified as a benign adenoma rather than a malignant interacinous adenoma as indicated by Nigrelli (*52*). Thyroid enlargements were noted in *C. variegatus* held in aquaria at our laboratory; histological examinations by one of us (Couch) indicated thyroid hyperplasia. The only other tumor in this species we found mentioned in the literature (*54*) is an odontogenetic tumor (table 2).

Thyroid tumors or hyperplasia similar if not identical to those described in *C. variegatus* have also been reported (table 2) in *F. heteroclitus* from the New York Aquarium (*52*).

In addition, 3 specimens of *F. grandis* with suspect multicolored chromatophoromas composed of cells containing mosaics of different pigment organelles (fig. 9) have been studied at our laboratory by Couch. The fish were obtained from a mariculture facility on the Gulf Coast of Alabama, and were the older, larger specimens of over 6,000 fish examined. Detailed light microscopy (figs. 10, 11), electron microscopy, chromatographic, and cell culture studies were conducted on the tumors. The tumor cells possessed apparent pterinosomes as the dominant cytoplasmic organelle, but many cells also possessed possible premelanosomes, melanosomes, and possibly melanized reflecting platelets. The major pigments isolated and identified were pteridine pigments. Black pigments presumed to be melanin (fig. 10) were observed by light and electron microscopy. No carotenoid pigments or carotenoid vesicles were identified or detected. Couch proposed that the tumors may have arisen from neoplastic stem pigment cells or from anaplastic pigment cells. The tumors were invasive (figs. 10, 11) and replaced and isolated normal muscle tissues, but they were not metastatic. Chromatophoromas of 1 specimen, which eventually died in the aquarium, grew and increased in numbers. The cause of the neoplasm in *F. grandis* is unknown, but both environmental and genetic factors may be involved in its genesis.

CARCINOGEN-INDUCED LESIONS

We have worked with *C. variegatus* for 3 years to determine its potential as a carcinogen assay organism in long- and short-term exposure in critical life-stage tests with suspect and known carcinogens. Furthermore, we and B. J. Martin, of the University of Southern Mississippi, have examined *C. variegatus* for its responsiveness to at least 3 carcinogens in recirculating test systems, using both water and feeding as exposure routes. A brief report of the results, to date, of tumor induction tests with 1) trifluralin, a herbicide, 2) benzidine, and 3) aflatoxin B_1 follows. Each test was designed and performed in different modes and for different purposes; for the most part, environmentally realistic exposure concentrations (low parts per billion range) were tested.

Trifluralin

Embryos and 30-day old postfertilization fry were exposed to 1–5 μg trifluralin/liter for 19 months in a test system previously noted. Although no invasive neoplasms were found in these fish, several lesions were formed as a result of the chronic exposure (*38, 55, 56*). Most significant was a high prevalence of diffuse vertebral dysplasia, focal hyperostosis in the backbone, and apparent pituitary enlargement and histopathological changes. The vertebral dysplasia was characterized by the abnormal persistence of osteoblastic cells that continued to form cartilage and bone. Although Couch et al. (*38, 55, 56*) found this to be a persistent cellular phenomenon (stimulated by trifluralin), perhaps combined with a minor cellular proliferative one, the end result was the production of focal benign growths in the vertebrae. Pathobiologists and biologists working in fisheries should be aware of this type of lesion and avoid confusing it with neoplasia, particularly in fish from feral populations, but also in those exposed to carcinogens in the laboratory.

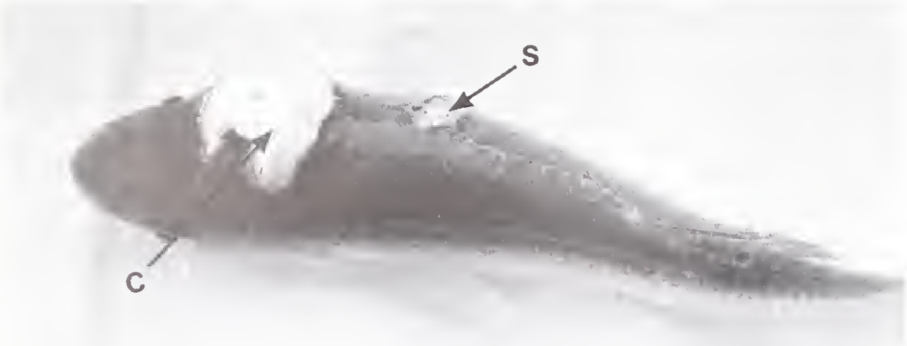

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FIGURE 9.—Adult *F. grandis* with suspect chromatophoroma (C) protruding from dorsal surface of anterior trunk. Secondary tumor (S) protrudes posterior to large tumor.

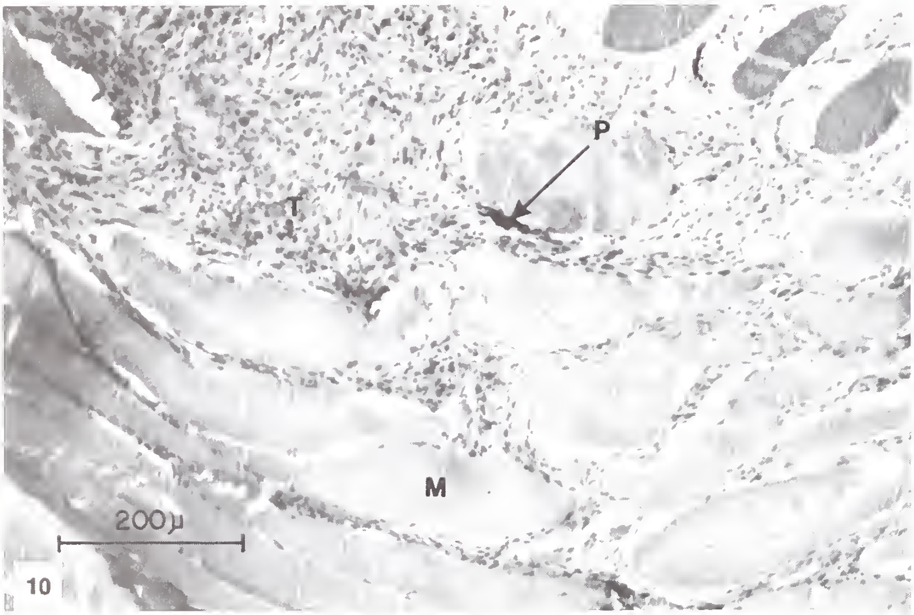

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FIGURE 10.—Tumor cells (T) invade muscle bundles (M), gradually surrounding and isolating them. Black pigments (P), presumed to be melanin, are deposited throughout the tumor site. Chromatographic analyses revealed pteridine pigment in these cells. H & E. × 125

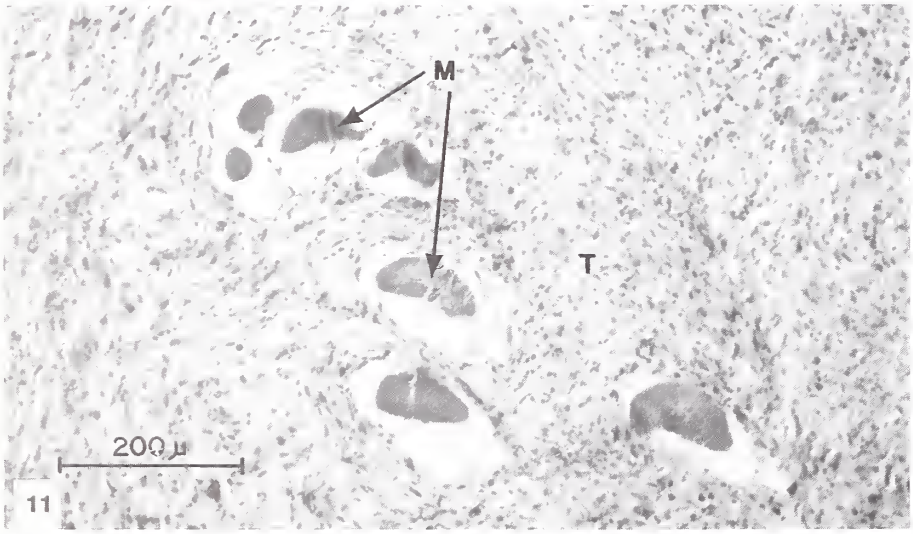

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FIGURE 11.—Muscle bundles (M) are completely isolated by invading tumor cells (T) and appear partially lysed. H & E. × 125

Benzidine

Apparent proliferative liver lesions were produced in *C. variegatus* exposed to 1 ppm benzidine dihydrochloride in recirculating systems (Martin BJ: Personal communication). Exposures were pulsed at 12-day intervals for 166 days and longer, at which time several fish became moribund. Histological examination revealed lesions involving tubular proliferation of either bile ductules or vascular tubules in the livers. Work continues on identification; however, preliminary results suggest that these lesions are hemangioendotheliomas, cholangiocarcinomas, bile duct adenomas, or merely adenofibrosis.

Aflatoxin B_1

To test the responsiveness of *C. variegatus* to an overt carcinogen, we exposed embryos and larvae (critical life stages) to aflatoxin B_1. This compound produces a high incidence of hepatocellular carcinomas in rainbow trout exposed as embryos to 500 ppb aflatoxin B_1 (*39*). The 96-hour-old embryos and 120-hour-old, newly hatched fry were exposed in a glove box under static conditions for 1 hour to 200 μg aflatoxin B_1/liter with dimethyl sulfoxide as a carrier. Embryos at this age were chosen because, at this stage, liver tissue, the apparent target tissue of aflatoxin B_1, is differentiated and well developed (figs. 5, 6); however, the embryos were encased in the chorion and penetration of the compound presented a possible problem. Therefore, we also exposed newly hatched fry (120 hr old) to circumvent the penetration problem. After exposure, the animals were transferred to static 200-liter holding aquaria equipped with under-substrate filters and maintained for 6 weeks. Then they were transferred to and are still being maintained in 38-liter aquaria with flowing water. Fish have been taken at 1 month, 3 months, and 6 months postexposure for histological examination. The most significant finding to date is a high incidence of mitotic figures in the hepatocytes of the exposed fish from the 1-month sample. Hepatic cell mitotic activity (fig. 12) nine times that of controls was noted in exposed fish. Later samples failed to reveal any significant lesions, nor was unusual mitotic activity noted. The fish continue to be maintained and additional sampling is scheduled for 12 months.

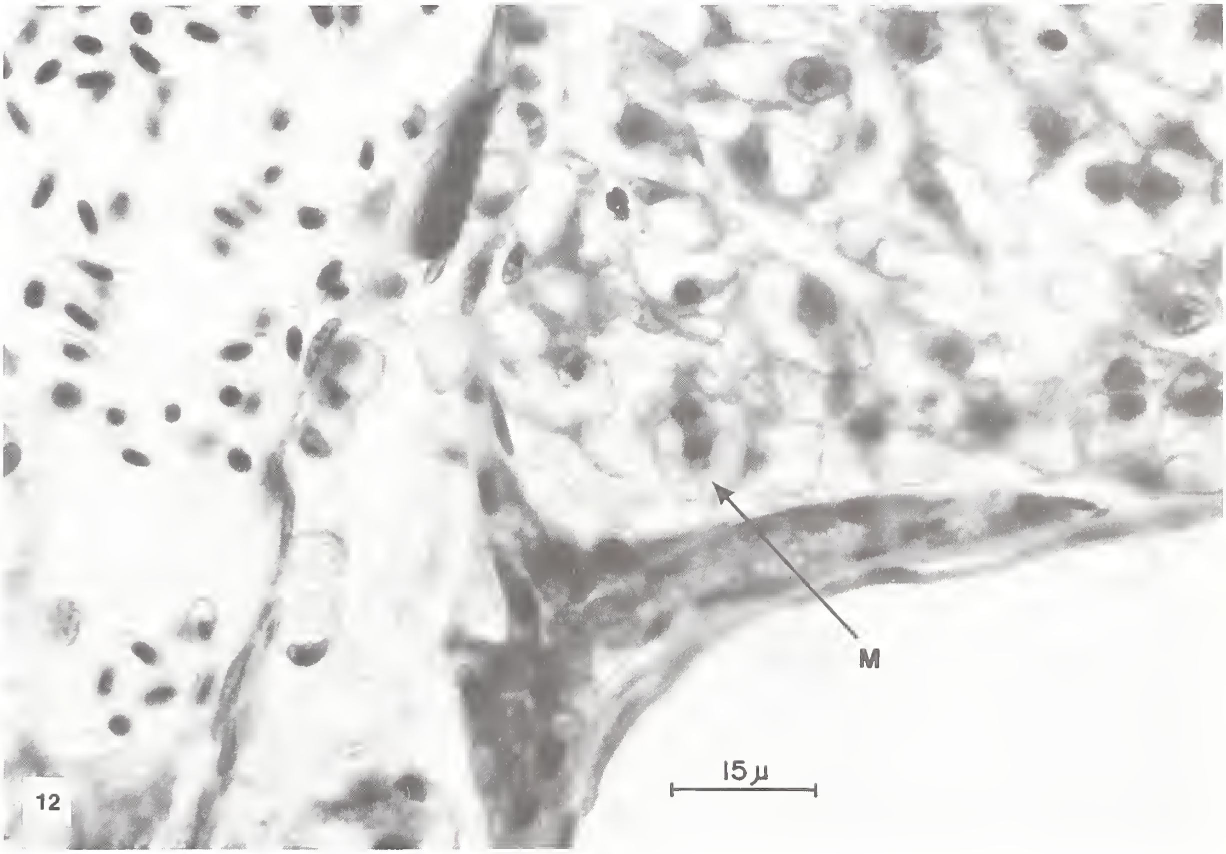

FIGURE 12.—Liver section from 1-mo-old *C. variegatus* exposed as 120-hr fry to 200 μg aflatoxin B_1/liter for 1 hr. *Note* mitotic figure (M). Mitotic activity nine times greater than control was observed in exposed specimens. H & E. × 1,325

ADVANTAGES AND PROBLEMS WITH *CYPRINODON* AND *FUNDULUS* AS CARCINOGEN TEST SPECIES

C. variegatus and *F. grandis* possess many assets that make them desirable test organisms. Although certain problems must be considered when they are used in cancer research, the advantages presented by *C. variegatus* and *F. grandis* far outweigh the possible problems encountered with their use; the potential of these species should be fully explored.

In summary, many advantages are exhibited by both species. These include: 1) wide geographic distribution; 2) abundant, ecologically significant estuarine species; 3) transportability; 4) easy culture and maintenance; 5) environmental adaptability (e.g., salinity, temperature); 6) typical teleostean anatomy and histology and thus adaptability to routine and special histological techniques; 7) easily fulfilled nutritional requirements; and 8) documented history of morphological and physiological responsiveness to toxic chemicals (especially with *C. variegatus*). Moreover, both species are euryhaline, but, in estuarine and marine water, they drink the seawater and thus expose the entire alimentary canal to a water-borne carcinogenic agent.

Each species has, in addition, its specific advantages. The *C. variegatus* offers 1) a small organism that required limited holding space, 2) short embryological development period, 3) short generation time, and 4) a large toxicological data base. *Fundulus*, on the other hand, offers 1) a larger organism that is more easily adaptable to certain special procedures (e.g., serum or injection studies), 2) extended developmental and generation periods, and 3) larger organ systems for use in biochemical studies.

Problems encountered with these species are, for the most part, also experienced with most other test animals. The fishes are susceptible to some infectious and noninfectious disease agents often encountered in the natural history and culture of teleost species. For example, monogenetic trematodes (*Gyrodactylus* spp.) are sometimes a problem with *C. variegatus*. *Eimeria funduli*, a pathogenic coccidial protozoon, infects *F. grandis* and may greatly affect growth (*57*). However, the disease problem with these species is not exceptional. We have encountered no major problems with disease in several years of study, and with control of extreme environmental fluctuations, these species appear less susceptible to culture-related diseases than many others.

An additional potential problem that is widespread with many fish species is the penetrability of the chorionic membrane by test compounds. To circumvent this problem, we are studying the usefulness of penetrants, such as dimethyl sulfoxide. Additionally, dechorionation methods (*58, 59*) may be feasible. Moreno (*58*), using *C. variegatus*, describes a method involving penetration of the chorion with the sharpened end of a pair of forceps and gently rolling the egg under the blade of a scalpel until the chorion is excised. This is essentially a shelling out process and can be performed with minimal damage to the embryo.

Of final and central importance is an apparent low sensitivity to suspect carcinogenic agents noted in our studies. The spectrum of agents tested thus far, however, has been limited, and we have purposely used environmentally realistic, low concentrations. Though neoplastic responses have been dubious, some suspect proliferative lesions have been induced with specific agents (e.g., benzidine induction of proliferative tubular elements in the liver of *C. variegatus*). More intensive and diverse experimentation with a broader spectrum and higher concentrations of suspect carcinogenic agents for the evaluation of the responsiveness of both species is warranted.

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DISCUSSION

J. D. Hendricks: What were the concentrations and times of exposure of *Cyprinodon* embryos to aflatoxin B_1?

J. A. Couch: We used 200 μg/liter for 1 hour for the 96- and 120-hour embryos.

A. Woodhead: What are the frequencies and significance of the aging pigments in the livers that you showed?

Couch: We find that livers from fish that are diseased, stressed, or exposed to toxic chemicals may have larger amounts of the hemosiderin or melanin-macrophage centers than do normal livers from healthy fish. Even some apparently healthy fish from nature have relatively large amounts of these pigments. They seem to be related to injury when they occur in large amounts.

G. Streisinger: Is it possible to collect ova and especially sperm without killing the animal, e.g., using capillary tubes to collect milt?

L. A. Courtney: Collection of ova from ripe females can and has been done with *Cyprinodon variegatus*. Dissection is usually used for two reasons: When human chorionic gonadotropin injections are used, the possibility of subsequent infection exists, and therefore the ova are not manually stripped. We use dissection instead. When human chorionic gonadotropin is not given, manual stripping works quite well, and females remain healthy and continue to produce. Collection of sperm from males by capillary tubing has not been done in our laboratory; however, I believe it could be done easily with either species (*Cyprinodon* or *Fundulus*).

Xiphophorus as an In Vivo Model for Studies on Oncogenes[1, 2]

Fritz Anders,[3] Manfred Schartl,[3] *and* Angelika Barnekow[4, 5]

ABSTRACT—The capacity of *Xiphophorus* to develop neoplasia can be formally assigned to a "tumor gene" (*Tu*), which appears to be a normal part of the genome of all individuals. The wild fish have evolved population-specific and cell type-specific systems of regulatory genes (*R*) for *Tu* that protect the fish from neoplasia. Hybridization of members of different wild populations in the laboratory followed by treatment of the hybrids with carcinogens led to disintegration of the *R* systems permitting excessive expression of *Tu* and thus resulting in neoplasia. Certain hybrids developed neoplasia even spontaneously. Observations on the genuine phenotypic effect of the derepressed *Tu* in the early embryo indicated an essential normal function of this oncogene in cell differentiation, proliferation and cell–cell communication. *Tu* appeared to be indispensable in the genome but may also be present in accessory copies. Recently, *c-src*, the cellular homolog of the Rous sarcoma virus oncogene *v-src*, was detected in *Xiphophorus*. The protein product of *c-src*, $\mathrm{pp60}^{c-src}$, was identified and then examined by its associated kinase activity. This $\mathrm{pp60}^{c-src}$ was found in all individuals tested, but, depending on the genotype, its kinase activity was different. The genetic characters of *c-src*, such as linkage relations, dosage relations, expression, etc., correspond to those of *Tu*. From a systematic study which showed that $\mathrm{pp60}^{c-src}$ was present in all metazoa tested ranging from mammals down to sponges, we concluded that *c-src* has evolved with the multicellular organization of animals. Neoplasia of animals and humans is a characteristic closely related to this evolution. Our data showed that small aquarium fish, besides being used successfully because they are time-, space-, and money-saving systems for carcinogenicity testing, are also highly suitable for basic studies on neoplasia at the populational, morphological, developmental, cell biological, and molecular levels. — Natl Cancer Inst Monogr 65: 97-109, 1984.

Neoplasia was found in all taxonomic groups of eumetazoa and was even detected in fossils of prehistoric animals, such as sauria, and mammals including humans (*1–3*).

Neoplasia occurs infrequently in the natural populations of eumetazoa, and induction by carcinogens is difficult (*4, 5*). However, certain nontaxonomically defined groups of animals are highly susceptible to both spontaneously developing and carcinogen-triggered neoplasms [table 1; (*6–31*)]. These groups consist mainly of animals of hybrid origin, such as naturally occurring or experimentally produced interspecific, interracial, and interpopulational hybrids (*5*) as well as laboratory and domesticated animals (*6*) which actually are also hybrids (i.e., homozygous combinations of chromosomes of different population or racial provenance). These animals share their high susceptibility to neoplasia with humans (*4*). The hybrid animals can serve as models for the research on the origin of neoplasia in humans and can serve as tester organisms for the carcinogens in our environment (*32*). The rationale underlying the use of these animals as models is based on the assumption that the biological fundamentals of neoplasia are similar in models and human beings.

Although mice and rats are the classic laboratory animals used in experimental cancer research, several genera of small teleost fish serve increasingly as models in new cancer research programs. Their suitability is beyond question (except for research on breast cancer, lung cancer, etc.), and their efficiency is more economic and time-saving as compared with that of the mammals (*32, 33*). One of these genera is *Xiphophorus,* the animal model that we have used in our laboratories for 25 years (*4, 5, 34–36*).

TAXONOMIC STATUS OF THE WILD *XIPHOPHORUS*

Xiphophorus, including platyfish and swordtails, lives in genetically isolated populations in brooks, rivers, lakes, ponds, and pools in Central America and has evolved into innumerable genotypically and phenotypically distinguishable groups. Based on certain morphological, ecological, and ethological characteristics, 17 of these groups have been listed as species (*37, 38*). All individuals of this genus, however, can be hybridized in the laboratory without difficulty, and all hybrids are fertile. This finding and those results on the conformity of genome organization (*39*), the low degree of enzyme polymorphism (*40*), and the normal chromosome pairing during meiosis in the hybrids (*41*) led to the conclusion that the taxonomic differences between these groups of *Xiphophorus* are not at the species level but at the level of elementary local populations as well as

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ABBREVIATIONS: MNU = *N*-methyl-*N*-nitrosourea; BC = backcross generation; *Tu* = tumor gene; *R* = pigment cell-specific regulatory gene for *Tu*; *v-src* = transforming gene from Rous sarcoma virus; *c-src* = cellular homolog of *v-src*; $\mathrm{pp60}^{v-src}$ and $\mathrm{pp60}^{c-src}$ = phosphoprotein gene products of *v-src* and *c-src* (mol wt = 60,000).

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Table 1.—*Animals that exhibited a high tumor incidence*

Species	Tumor	Reference
Insects		
Drosophila laboratory stocks	Various neoplasms	(*7*)
Solenobia hybrids	" "	(*8*)
Teleosts		
Xiphophorus hybrids	" "	(*9–11*)
Girardinus laboratory stocks	Promoter-induced melanoma	(*12*)
Ornamental guppy strains	Carcinogen-induced hepatoma	(*13*)
Orange medaka	Hepatoma	(*14*)
Domesticated trout	Aflatoxin-induced liver tumors	(*15*)
Salvelinus hybrids	Fibrosarcoma	(*16*)
Domestic carp	Neuroepithelioma	(*17*)
Ornamental hybrid carp	Ovarian neoplasia	(*18*)
Lake Ontario hybrid carp	Pollution-conditioned gonadal tumors	(*19*)
Goldfish	Erythrophoroma	(*20*)
Amphibia		
Bufo calamita and *B. viridis* hybrids	Chordomas	(*21*)
Birds		
Musk duck and mallard hybrids	Gonadal tumors	(*22*)
Peacock and guinea fowl hybrids	" "	(*23*)
Improved breeds of fowl	Leukosis	(*6*)
Mammals		
Mus musculus and *M. bactrianus* hybrids	Various neoplasms	(*24*)
Laboratory mice strains	" "	(*25*)
Hybrids of mice strains	Increased incidence of various neoplasms	(*25*)
BALB/c and NZB hybrids	Plasma cell tumors (50%)	(*26*)
Blue ribbon mice	Mammary tumors (100%)	(*27*)
Sprague-Dawley and Long-Evans hybrids	Increased mammary tumor incidence	(*28*)
Domestic dogs	Various neoplasms	(*6*)
Boxers	" "	(*29*)
Domestic cats	" "	(*6*)
Sinclair swine	Melanoma	(*30*)
Lipizzaner horses	"	(*31*)

ecological and geographical races. The ease of hybridizing animals from the different taxonomic groups of *Xiphophorus* listed as species, therefore, is no curiosity in the animal kingdom but is comparable to the frequently occurring hybridization between animals of different populations or local races from other teleosts as well as amphibia, lizards, birds, mammals (*40*), and humans.

SUSCEPTIBILITY TO NEOPLASIA IN *XIPHOPHORUS* HYBRIDS

About 10,000 specimens of purebred descendants of the wild populations have been treated with powerful carcinogens, such as benzo[*a*]pyrene, MNU, and X-rays, but none developed neoplasia. Contrastingly, of 10,195 hybrids which survived treatment with MNU or X-rays, 805 (8%) of the individuals developed a large variety of neoplasms (table 2). Most of the neoplasms were classified as neurogenic and mesenchymal, with melanoma, neuroblastoma, and fibrosarcoma the predominant types. Epithelial neoplasms were less frequent but comprised those with the largest diversity. Tumors comparable to those of mammals and humans developed in almost all tissues including connective tissue, muscles, and the pigment cell, nervous cell, gastrointestinal, and reticuloendothelial systems.

ASSIGNMENT OF CANCER SUSCEPTIBILITY TO ONCOGENES AND REGULATORY GENES

At present, *Xiphophorus* is the only model, at least to our knowledge, in which susceptibility to carcinogen-triggered neoplasia was assigned to oncogenes and regulatory genes. Information about these genes comes from the analysis of the hereditary trait of certain spontaneously developing melanomas, pterinophoromas, neuroblastomas, thyroid carcinomas, and reticulosarcomas in hybrids. The hybrid animals that develop the neoplasms spontaneously are rare compared with those that require carcinogen induction for tumor development. However, they can be bred at the will of the experimenter and have therefore contributed many facts for development of ideas about how other animals and humans might inherit susceptibility to neoplasia (*34–36, 46, 47*).

Oncogenes and Regulatory Genes in Animals Developing Melanoma Spontaneously

To illustrate the assignment of cancer susceptibility to distinct genes in greater detail, we used melanoma because this type of tumor is easily detectable by its heavy melanin pigmentation. Even singly transformed pigment cells can be distinguished from nontransformed pigment cells by gross

TABLE 2.—*Neoplasia in* Xiphophorus F_2–F_{24} *and* BC_n *generations*[a]

Type of neoplasm	No. of neoplasms			Percent incidence based on total No. of survivors	
	MNU	X-rays		MNU	X-rays
Neurogenic					
Melanoma (benign)	135	93	491	2.12	2.6
Melanoma (malignant)	138	34		2.09	0.95
Neuroblastoma	84	7		1.27	0.2
Epithelial					
Squamous cell carcinoma	6	0	78	0.09	0
Epithelioma	19	6		0.28	0.17
Carcinoma (low differentiation)	3	4		0.05	0.11
Carcinoma (high differentiation)	2	5		0.03	0.14
Adenocarcinoma (kidney)	8	2		0.12	0.05
Adenocarcinoma (thyroid)	2	3		0.03	0.08
Papilloma	9	0		0.14	0
Hepatoma	5	1		0.07	0.03
Acanthoma	3	0		0.04	0
Mesenchymal					
Fibrosarcoma	190	6	236	2.87	0.17
Rhabdomyosarcoma	33	2		0.5	0.05
Lymphosarcoma	1	0		0.01	0
Reticulosarcoma	4	0		0.06	0
Total	642	163	805		

[a] One year after treatment with MNU (10^{-3} *M* four times for 1 hr in 2-wk intervals) and X-rays (1,000 roentgens three times for 45 min in 6-wk intervals). Survivors: MNU=6,608; X-rays=3,587. Data were compiled from (*42–45*).

examination of the living animal (*48*). Furthermore, morphological, ultrastructural, and histochemical markers specific to the successive stages of pigment cell differentiation facilitate the detection of the event of neoplastic transformation. This facilitation and the ease with which crossings in *Xiphophorus* can be accomplished provided the background for the design of the experiments that elucidated the oncogenes and regulatory genes by formal genetics.

Crosses (fig. 1A–H) of a certain *X. maculatus* (platyfish), which is infrequently spotted (A), with a certain *X. helleri* (swordtail), which is never spotted (B), result in F_1 that develop uniformly benign melanoma instead of spots (C). Backcrosses of the first generation hybrids with the swordtail as the recurrent parent (D) result in offspring (BC_1), 50% of which exhibit neither spots nor melanoma (G and H), whereas 25% develop benign melanoma (E), and 25% develop malignant melanoma (F). Further backcrosses (not shown in fig. 1) of the fish carrying benign melanoma with the swordtail result in a BC_2 that exhibits the same segregation as the BC_1. The same applies for further backcrosses of this kind. Backcrossing of the fish carrying malignant melanoma with the swordtail results, however, in a BC_2 in which 50% of the animals do not develop melanoma, whereas the remaining 50% develop malignant melanoma. In contrast, backcrossing of the melanoma-bearing hybrids with the use of the platyfish as the recurrent parent results in a gradual suppression and finally disappearance of neoplasia in the succeeding generations (fig. 2).

The results obtained in these 2 series of crossing experiments revealed several genetic components that are involved in melanoma formation or protection from melanoma (*see* fig. 3):

1) The platyfish genome contains the genetic information for the development of spots which is lacking in the swordtail. Because the spots consist of transformed pigment cells (*48*), this information is considered to be encoded by an oncogene, *Tu*, which is an accessory gene of the platyfish genome. From about 70 structural changes involving crossovers, deletions, duplications, and translocations, we know that *Tu* is normally located at the end of the X-chromosome of *X. maculatus* (*42, 49–51*) and is apparently also responsible for the large variety of the carcinogen-triggered neurogenic, epithelial, and mesenchymal neoplasms (*35, 43*) listed in table 2.

2) The protection of the platyfish from the activity of its own *Tu* is apparently exerted by regulatory genes. After hybridization, *Tu* becomes dysregulated indicating that the swordtail lacks not only the oncogene *Tu* but also the regulatory genes (*34*). Several types of regulatory genes can formally be deduced from the outcome of the crossing experiments: 1) The restriction of crossing-conditioned neoplasia to melanoma indicates the presence of a *Tu*-linked pigment cell-specific regulatory gene which, as we know from mutagenesis studies (*35, 42, 50, 51*) and from comparison of the *Tu*-containing chromosomes shown in the schemes of figs. 3 and 4 (*see* later), is impaired by mutation. Out of the tissue-specific regulatory genes only the impaired pigment cell-specific regulatory gene for *Tu*, i.e., *R′*, is shown in the scheme (fig. 3). 2) The restriction of the crossing-conditioned melanomas to the posterior part of the body and to the dorsal fin indicates the presence of *Tu*-linked regulatory genes that are specific to certain compartments of the body. In additional experiments corresponding to those shown in figs. 1–3, depending on the genotype (mutant) of the platyfish used for the initial crosses, melanoma develops, for instance, in the anterior part of the body, anal fin, tail fin, mouth, eye, peritoneum, or meninx, etc. Thirteen compartments have been identified that correspond to different genes which, in turn, correspond to sites of the body where the melanomas occur (*35*). Out of these compartment-specific regulatory genes only the impaired dorsal fin-specific regulatory gene, i.e., $R_{Df'}$, and the posterior part-specific regulatory gene, i.e., $R_{Pp'}$, are shown in the scheme. 3) The clear-cut 1:1 segregation between the BC-hybrids bearing malignant melanomas and those bearing benign melanomas indicates the existence of a prominent regulatory gene that is not linked to the oncogene *Tu*. Because the benign melanomas consist, in contrast to malignant melanoma, predominantly of terminally differentiated transformed pigment cells (*48, 52*) this regulatory gene is considered to be involved in differentiation of these cells, and was, therefore designated

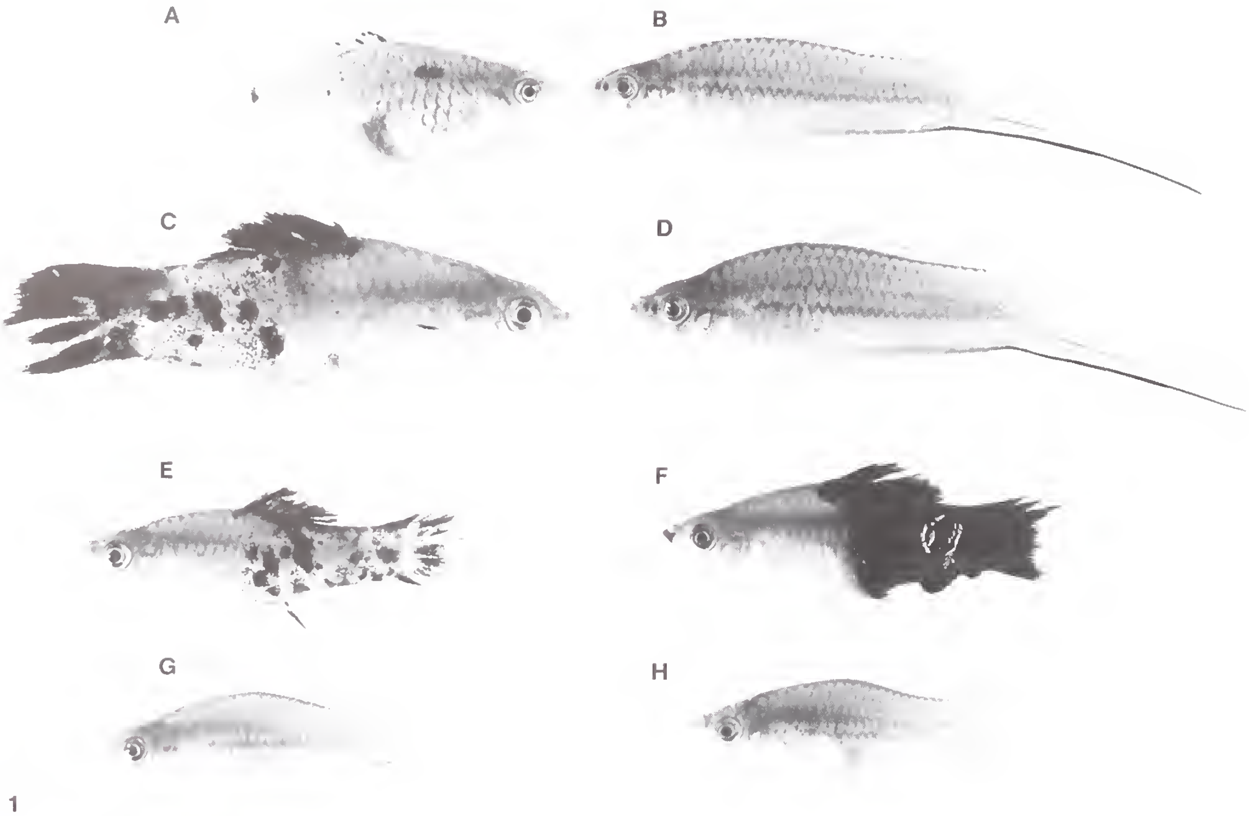

FIGURE 1.—Crossing procedure for the production of melanoma developing hybrids of *Xiphophorus*. A) *X. maculatus* from Rio Jamapa (Mexico); some small spots in the skin of the dorsal fin and the side of the body are visible. Spots consist of terminally differentiated, neoplastically transformed pigment cells. B) *X. helleri* from Rio Lancetilla (Mexico) always lacked spots. C) F_1 developed benign melanoma instead of spots (100% of the F_1). D) *X. helleri* from B) used in the backcross as the recurrent parent. E) Backcross hybrid with benign melanoma (25% of the BC generation). F) Backcross hybrid with malignant melanoma (25% of the BC generation). G), H) Backcross hybrids that do not develop melanoma (50% of the BC generation).

as *Diff*. The *Diff*-containing chromosome can easily be detected by an esterase marker, *Est-1*, which is closely linked to *Diff* (*35; 53–59*). Additional genes involved in melanoma formation have been identified but have not been taken into consideration in this study.

Following crossings and backcrossings with the swordtail as the recurrent parent, the chromosomes of the platyfish are replaced by the homologous chromosomes of the swordtail, resulting in the gradual disintegration of the regulatory gene system for *Tu*. Thus the *Tu* hybrids develop spontaneously benign melanoma if some regulatory genes such as *Diff* are still present in the system and malignant melanoma if the regulatory genes are lacking (*34, 35, 53, 56, 58, 59*). If *Tu* is lacking, no melanomas occur.

In contrast, after backcrossings of the melanoma-bearing hybrids with the platyfish as the recurrent parent (fig. 2), the chromosomes carrying regulatory genes are reintroduced into the descendants. This results in a reconstruction of the original regulatory gene system that suppresses the activity of *Tu*.

Oncogenes and Regulatory Genes in Animals Requiring Carcinogenic Inducers for the Development of Melanoma

To relate the genes that are responsible for the spontaneous development of melanoma to the genes that are basically involved in the development of the carcinogen-dependent neoplasms, we replaced the R' R_{Df}' R_{Pp}' *Tu*-chromosome (fig. 3) by the R R_{Df} R_{Bs}' *Tu*-chromosome (fig. 4), the pigment cell-specific R and dorsal fin-specific R_{Df} of which are nonmutated and active, and the compartment-specific R_{Bs} that normally protects the entire side of the body from melanoma is mutated to R_{Bs}'. The R_{Pp} was not taken into consideration in these experiments. Because R is inherited with *Tu*, melanoma does not develop spontaneously in the hybrids. After treatment with carcinogens, those hybrids carrying the R R_{Df} R_{Bs}' *Tu*-chromosome but lacking the nonlinked regulatory genes including *Diff* are highly sensitive to the carcinogens on the body side. Thus development of melanoma requires only impairment or deletion of the pigment cell-specific R gene

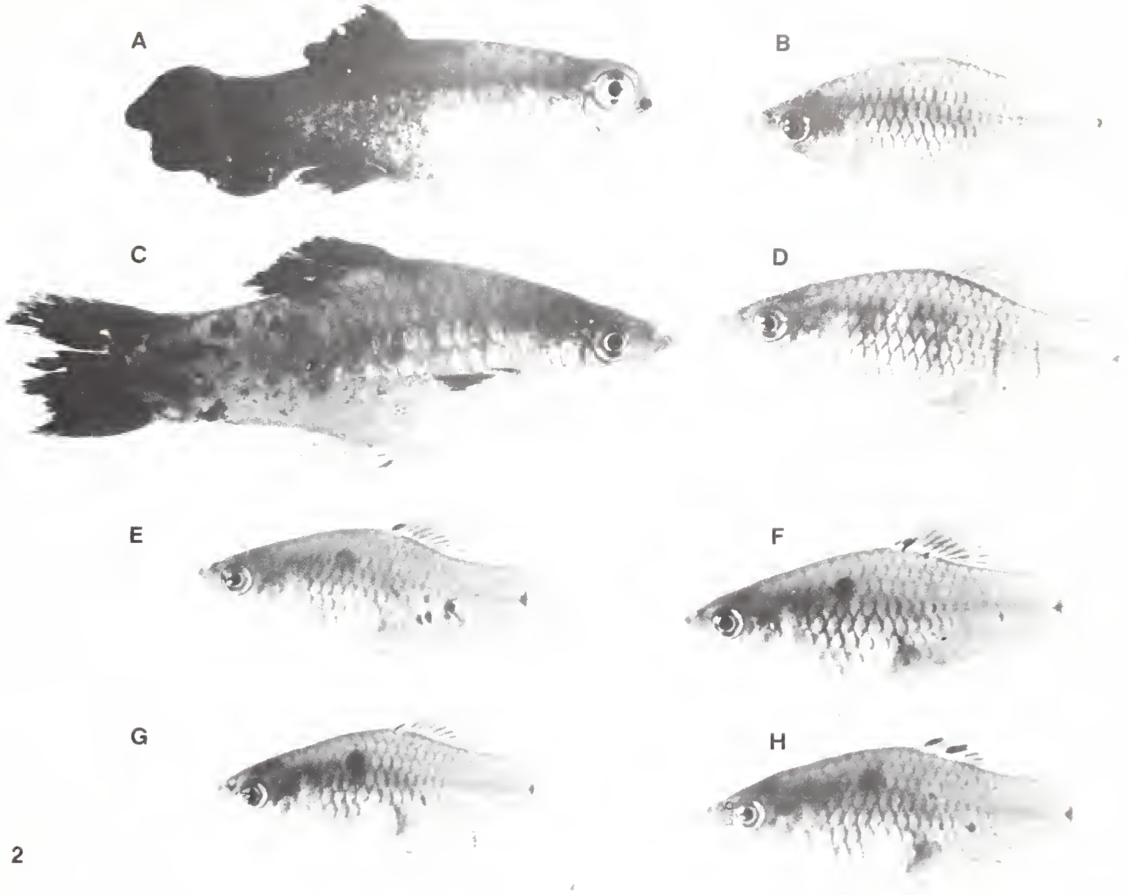

FIGURE 2.—Crossing procedure for the suppression of melanoma in *Xiphophorus*. A) Malignant melanoma bearing backcross hybrid according to figure 1F. B) *X. maculatus* according to figure 1A as the recurrent parent. C) Quasi F_1 exhibiting benign melanoma. D) *X. maculatus* as the recurrent parent. E), F), G), H) Backcross hybrids (quasi *X. maculatus*) exhibiting spots only.

by the carcinogen in a somatic cell at the side of the body (*32, 60, 61*).

Problem of Tissue Specificity of the Oncogene and of the Regulatory Gene System

The hybrid segregants that were highly susceptible to carcinogen-triggered melanoma were also susceptible to the induced neuroblastoma, epithelioma, and fibrosarcoma (*43-45, 60, 61*). Furthermore, many hybrid individuals treated with carcinogens, in addition to the melanoma, may also develop multiple neoplasms, such as neuroblastoma, retinoblastoma, carcinoma, and sarcoma. Therefore, the development of the different carcinogen-induced neoplasms apparently depends on the same *Tu*-containing chromosome like that of melanoma (*43-45, 60, 61*). Additional studies are required before investigators can decide whether 1) *Tu* is tissue-nonspecific although tissue specificity of the tumor comes from *Tu*-linked, tissue-specific *R* genes, or 2) whether *Tu* is an oncogene complex composed of tissue-specific constituents that are under the control of linked *R* genes specific to the *Tu* constituent. Experiments in which susceptibility to neurogenic and epithelial neoplasia could be assigned to a certain chromosome, though susceptibility to neoplasms of mesenchymal origin depended on a different chromosome (*60, 61*), tempt one to assume that *Tu* actually could be composed of several constituents that are tissue specific and could be considered as different oncogenes.

GENUINE ONCOGENIC EFFECT OF *Tu* IN THE PIGMENT CELL SYSTEM OF EARLY EMBRYOS

Information about the genuine oncogenic effect of *Tu* comes from a balanced laboratory stock carrying a lethal *Tu* translocation. The *Tu* copy became translocated from an X-chromosome of *X. maculatus* to an autosome of *X. helleri* and, in its new position, is no longer controlled by its formerly linked regulatory genes that act in *cis*-position only (*42*). Nonlinked regulatory genes are not present in the system except for *Diff*. The *Tu*-carrying progeny of the stock segregates into 50% carrying *Diff* which survive, whereas the corresponding 50% lacking *Diff* are lethal (*42*). As a consequence of the Mendelian inheritance of the

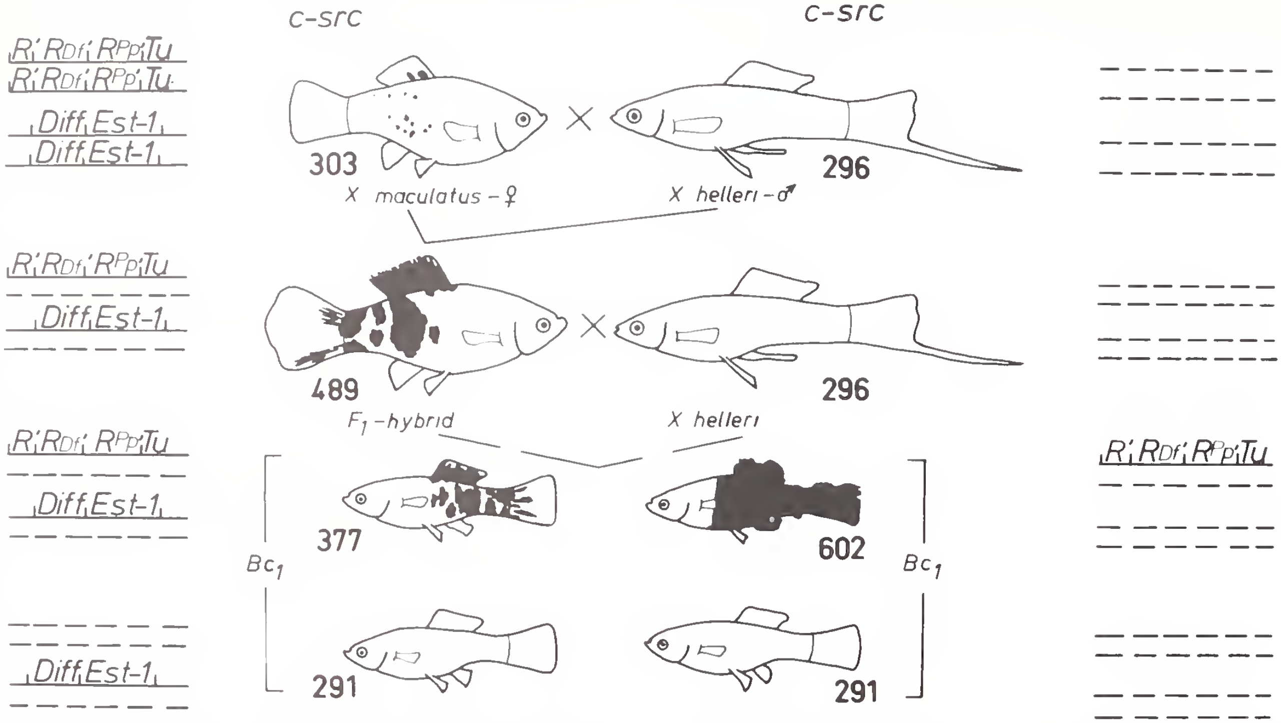
c-src
c-src
R' RDf' RPp' Tu
R' RDf' RPp' Tu
Diff Est-1
Diff Est-1
303
×
296
X maculatus - ♀
X helleri - ♂
R' RDf' RPp' Tu
Diff Est-1
489
×
296
F1-hybrid
X helleri
R' RDf' RPp' Tu
Diff Est-1
377
602
Bc1
Bc1
R' RDf' RPp' Tu
Diff Est-1
291
291

FIGURE 3.—Crossing scheme according to figure 1, which displays the genetic conditions for the "spontaneous" development of spots, benign melanoma, and malignant melanoma. ——— = chromosomes of *X. maculatus*; - - - - = chromosomes of *X. helleri*; *Tu* = tumor gene; $R_{Pp'}$ and $R_{Df'}$ = impaired regulatory genes controlling *Tu* in the compartments of the posterior part of the body (Pp) and the dorsal fin (Df); R' = impaired regulatory gene specific to pigment cells but nonspecific to the compartments; *Diff* = regulatory gene controlling differentiation of neoplastically transformed cells; *Est-1* = locus for esterase-1 of *X. maculatus*. *c-src* = pp60$^{c\text{-}src}$ kinase activity expressed as counts per minute/milligram protein); *note* basic and excessive activity and correlation between *c-src* expression and *Tu* expression. Data from (*4, 5, 32, 75*) were combined.

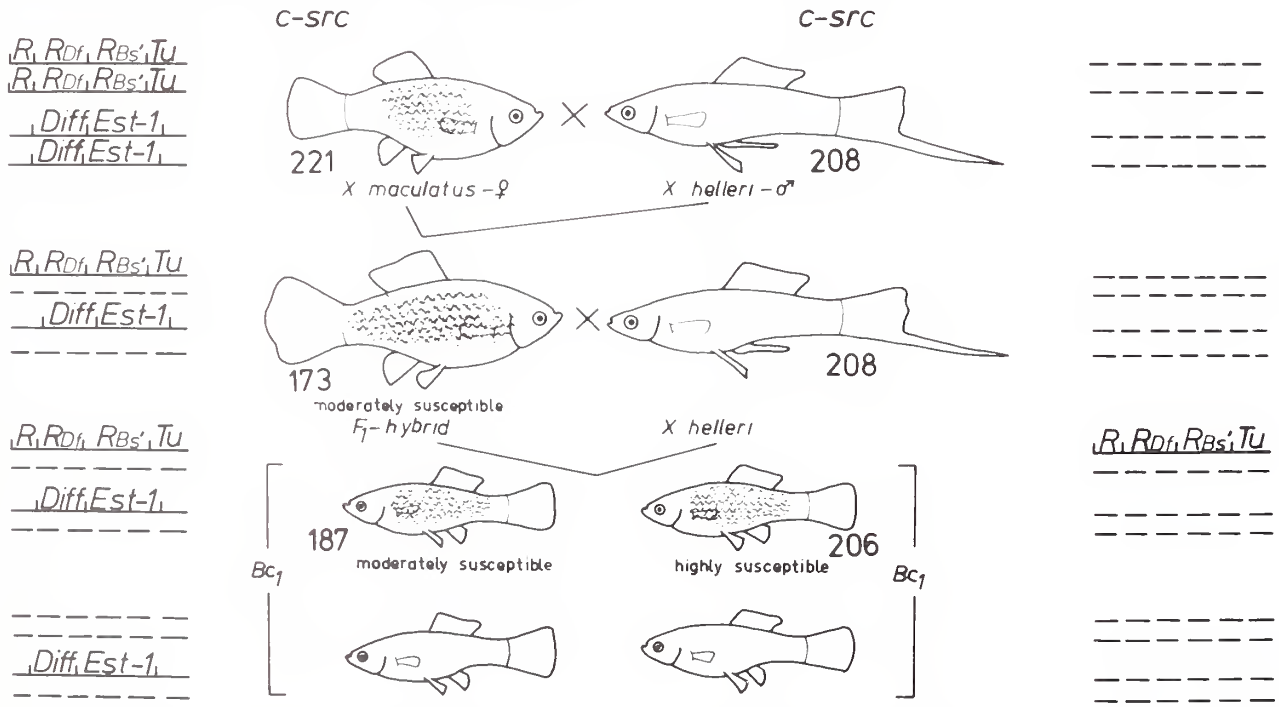
c-src
c-src
R RDf RBs' Tu
R RDf RBs' Tu
Diff Est-1
Diff Est-1
221
×
208
X maculatus - ♀
X helleri - ♂
R RDf RBs' Tu
Diff Est-1
173
×
208
moderately susceptible
F1-hybrid
X helleri
R RDf RBs' Tu
Diff Est-1
187
moderately susceptible
206
highly susceptible
Bc1
Bc1
R RDf RBs' Tu
Diff Est-1

FIGURE 4.—Crossing scheme displaying the genetic conditions for susceptibility to carcinogen-dependent neoplasia. The highly susceptible genotype is extremely sensitive to the carcinogenic (mutagenic) trigger. *See* legend to figure 3 for explanation of abbreviations. R_{Bs}' = impaired regulatory gene controlling *Tu* in the compartment of the entire side of the body (Bs). Data from (*4, 5, 32, 75*) were combined.

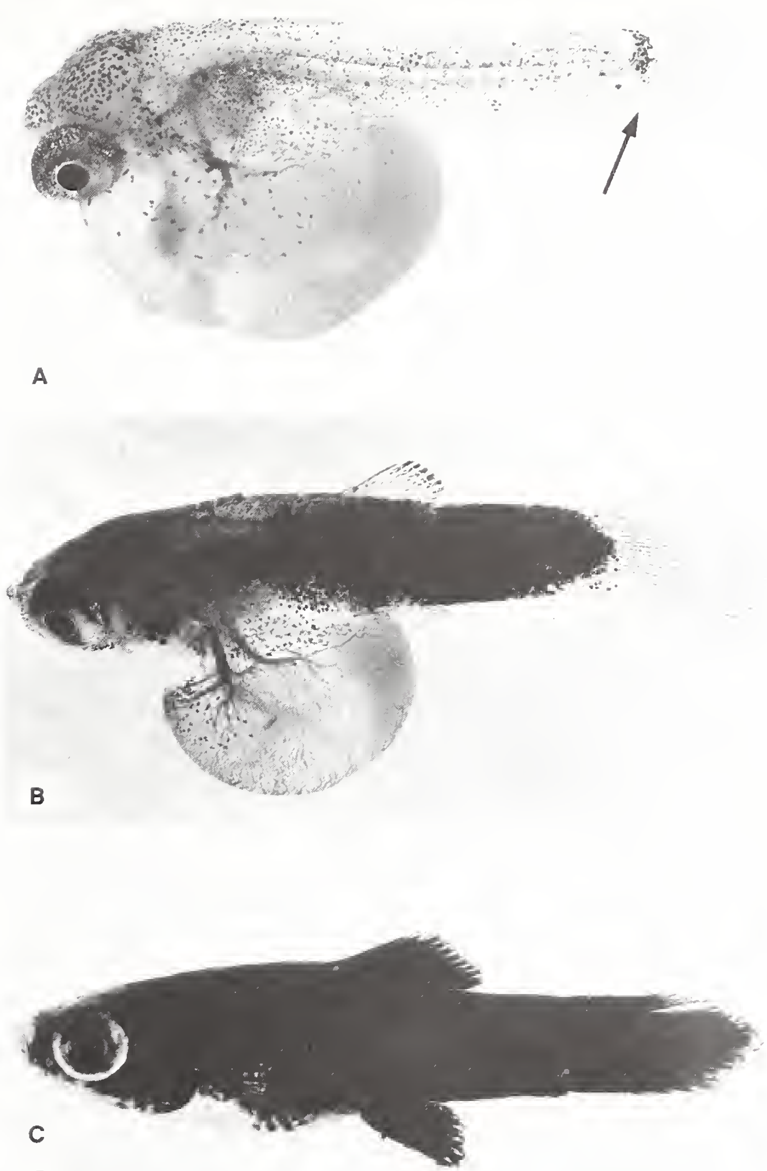

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FIGURE 5.—Genuine oncogenic effect of the tumor gene *Tu*. A) Ten-day-old embryo (3 mm long) exhibiting some T-melanocytes at the peduncle of the tail fin (*see arrow*). B) Same fish, 5 days later (4 mm long). C) Neonate of the same genotype (6 mm long).

unrestrained *Tu* through the germ line, the pigment cell precursors become transformed as soon as they become competent for neoplastic transformation by cell differentiation in the embryo (*62*). During the first days of embryonic life, differentiation of pigment cells still undergoes the normal course. After the embryo is 5 days old, some single cells become transformed, and at a later time about 10–20 dividing transformed melanoblasts appear in the peduncle of the tail fin. These melanoblasts differentiate within approximately 15 hours to transformed melanocytes (fig. 5A), which represent the predominant cells of the growing melanoma. During further development of the embryo, neoplastic transformation continues in all areas where pigment cell precursors become competent (fig. 5B), and the melanoma grows by both transformation and proliferation, thus developing into a "whole body melanoma" (fig. 5C), which will kill the fish before or after birth. The development of melanoma in the early embryo reflects the genuine oncogenic effect of the completely derepressed *Tu* on the pigment cell system (*35, 42, 62*).

CONCEIVABLE NONONCOGENIC FUNCTIONS OF *Tu* IN THE EMBRYO

The observations on the genuine oncogenic effect of *Tu* in the embryo tempt us to assume that *Tu* exerts important normal functions in cytodifferentiation and proliferation in the early embryo that are related to the neural crest where the pigment cell precursors originate. Moreover, we assume that in normal embryogenesis these functions become switched off or choked by the regulatory genes before the fifth day of embryonic life. If, however, the regulatory genes (i.e., the entire switch in the lethal *Tu* translocation) are lacking, *Tu* continues to exert its early embryo-specific functions which, as an extension of the cellular development in the early embryo, appear as transformation of the competent cells to the neoplastic state.

The assumption of normal (nononcogenic) functions of *Tu* in early embryogenesis raises the question regarding the genes that might exert these functions in the animals lacking *Tu*, such as swordtails, used in the experiments described earlier. This question leads to the problem of indispensable and accessory copies of the oncogenes that will be discussed later.

PROBLEM OF INDISPENSABLE AND ACCESSORY COPIES OF *Tu*

About 30 deletions of *Tu* have been genetically characterized, and some of the major deletions involving both *Tu* and its linked regulatory genes were, in addition to the genetic results, observed cytologically (*50*). Furthermore, deletions of *Tu* copies have been observed in all of our *Tu*-containing stocks of the genus *Xiphophorus*, including *X. maculatus*, *X. variatus*, *X. xiphidium*, *X. helleri* and *X. montezumae* (*42*). All deletions, even the loss of one *Tu*-containing Giemsa band in the homozygous condition in the female or in the heterozygous state in the male (observed in the X-chromosome of *X. maculatus*) have no detectable effect on the viability of the fish (*50*). This observation and the fact that wild populations may lack *Tu* in total led us to the conclusion that the *Tu* considered so far is accessory for the fish. We do not imply that the accessory *Tu* is lacking normal functioning. One could, for instance, assume that certain indispensable copies of *Tu* are present in the genome and may compensate the loss of the accessory *Tu* loci according to a gene dosage compensation mechanism, which warrants normal functions. Support for this assumption comes from the following experiment. Platyfish carrying the X-chromosome deletion of the Giemsa band that involves the accessory *Tu* were crossed with the swordtail according to the procedure outlined in figure 3. As was expected, no X-chromosome inherited tumors developed spontaneously in the hybrids, but after treatment with MNU, the backcross hybrids developed melanomas that could be assigned to an autosome of *X. maculatus*. Thus it appears that the platyfish, besides the easily detectable accessory *Tu* copies, also contains indispensable ones that require more intricate experiments for their detection.

So far we could not detect any individual of the genus *Xiphophorus* that lacks the genetic information for neoplastic transformation. Even individuals having lost the easily detectable *Tu* copies still contain oncogenes for the development of neoplasia. These oncogenes can always be noted after the appropriate crosses and with the appropriate carcinogenic treatment that trigger the neoplasms that are always chromosome specific. Therefore, oncogenes seem to be indispensable for the fish. Accessory copies of the oncogenes may be present but are not essential. If they are present, special regulatory gene systems are required for their control. We introduced up to 10 authentic copies of *Tu* (4 copies in homozygous duplications in both X-chromosomes of the female, 6 copies in 3 nonhomologous pairs of autosomes) and their linked regulatory genes into the genome containing nonlinked regulatory genes, but no tumors developed spontaneously nor could any effect on viability be observed. We assume that the *Tu* copies present in a certain genome are not strongly limited in number if their control is warranted by regulatory genes.

ONCOGENE DOSAGE EFFECT AND COMPENSATION

The following experiments illustrate the conditions leading to oncogene dosage effect and compensation. Both the X-chromosome of *X. maculatus* containing the accessory *Tu* (the X-chromosome according to fig. 3) and the X-chromosome of *X. maculatus* having lost the Giemsa band carrying the accessory *Tu* were introduced into the genome of *X. helleri* lacking both the accessory *Tu* and the regulatory gene system. The $X^{Tu}X^{Del} \times X^{Tu}$ Y-matings were accomplished. The segregating offspring having none, 1, or 2, respectively, accessory *Tu* copies showed a clear-cut gene dosage effect (fig. 6). The animals lacking *Tu* show no neoplastically transformed pigment cell (A), the littermates containing 1 *Tu* copy exhibit a limited number of transformed cells (B), and the segregants having inherited the double dosage of *Tu* develop malignant melanoma (C). However, if the experiment was modified by the use of animals as recipients for the X^{Tu}, X^{Del}- and Y-chromosomes that have retained the nonlinked regulatory genes, we observed gene dosage compensation (*63*). Dosage effect and compensation of the accessory oncogene *Tu*, therefore, depend on the absence or presence, respectively, of the nonlinked regulatory genes in these experiments. Oncogene dosage effect and compensation have been observed in many experiments of this kind (*63*).

CELLULAR COUNTERPARTS OF RETROVIRAL ONCOGENES IN THE *Tu* MELANOMA SYSTEM

Although neoplasia in *Xiphophorus* is well understood with regard to formal genetics, thus far its molecular basis has been extremely resistant to any elucidation.

Ten years ago, *Tu* of *Xiphophorus* was considered formally equated with oncogenes of endogenous tumor viruses (*42, 64*) that were interpreted in the sense proposed by Bentvelzen (*65*), Todaro and Huebner (*66*), and others (*67*). Our (*68*) experimental examination of this hypothesis, however, showed that B- and C-type particles present in melanoma and neuroblastoma following induction with 5-bromodeoxyuridine apparently are not related to neoplasia of the fish, and efforts to detect reverse transcriptase proved fruitless. This absence of prospects changed immediately when cancer virology progressed to the elucidation of cellular oncogenes.

Oncogene *v-src* from Rous sarcoma virus has a counterpart, *c-src*, in noninfected cells of the chicken (*69*). This *c-src* was also found in the human being, mouse, and calf (*70*). Commercial DNA derived from salmon obviously contains the same gene (*70*). However, no convincing evidence exists that relates the cellular *src* or its gene product, a 60,000-dalton phosphoprotein ($pp60^{c-src}$), with a

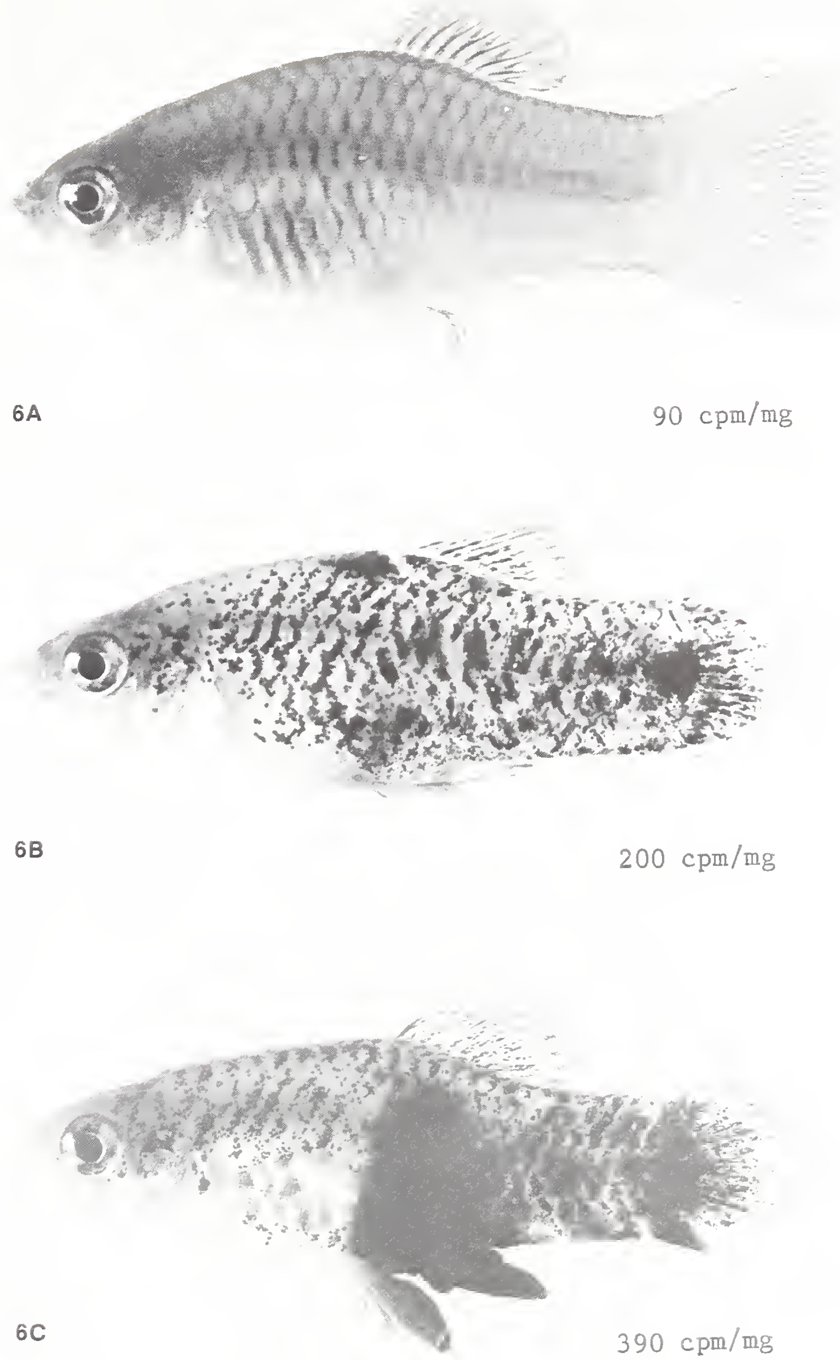

FIGURE 6.—Correlation between gene dosage effect of *Tu* (specified as the phenotype of the tumor) and pp60^{c-src} kinase activity (expressed as counts per minute/milligram protein) in littermates containing A) no accessory *Tu*, B) single dose of *Tu*, and C) double dose of *Tu*. Genetic background of the fish is identical. A) *Tu* is deleted in the germ line. B), C) The pigment cell-specific *R* linked to *Tu* is impaired by germ-line mutation.

tyrosine phosphorylating kinase activity to neoplasia that depends on conditions other than virus infections (*71, 72*). On the other hand, evidence had been presented that pp60^{c-src} is expressed in a differentiation-dependent and tissue-specific manner (*71*). This was the state of knowledge about cellular oncogenes when we started our search for *c-src* and its product pp60^{c-src} in *Xiphophorus*.

The *c-src* was detected in *Xiphophorus* by molecular hybridization of *src*-specific probe from cloned *v-src* with DNA from fish (Czernilofsky AP, Schartl M: Unpublished observations). To identify pp60^{c-src}, they labeled brains of the fish with [^{32}P]orthophosphate; the cell extracts were immunoprecipitated with antisera from Rous sarcoma virus tumor-bearing rabbits followed by polyacrylamide gel electrophoresis. The 60,000-dalton protein detected in the gel has a tyrosine-specific kinase activity and represents the pp60^{c-src} (*73*). The kinase activity was measured according to Collett and associates (*74*). For a detailed description of the experimental procedures, *see* the reports of Barnekow et al. (*73*), Schartl et al. (*75*), and Schartl and Barnekow (*76*). The experiments are based on the assumption that the activity of the pp60^{c-src}-associated phosphokinase monitors the activity of the endogenous *c-src* oncogene in *Xiphophorus.*

The kinase activity was determined in several tissues including skin, liver, spleen, testes, brain, and melanoma (*73*). The latter two always had the highest kinase activity, and genotype-specific differences in kinase activity were similar in both (*73*). Hence we could determine pp60^{c-src}-associated protein kinase activity mainly in brain extracts and relate the activity observed to the expression of *Tu* ascertained by the development of melanoma (*75, 77*). The possibility that the differences in kinase activity measured in the fish of different *Tu* genotypes are due to epiphenomena of the melanoma appears unlikely (*75*) because our results reflect the actual genetic activity of the *c-src* oncogene in the nontumorous brain tissue of the tumorous and nontumorous fish.

To study the possible relation between *Tu*-conditioned neoplasia and *c-src* expression, we took advantage of the 3 genetic experiments outlined in figures 3, 4, and 6.

In the experiment recorded in figure 3, the purebred *X. maculatus* carrying 2 repressed copies of the accessory *Tu*, as well as the purebred *X. helleri* and the BC-hybrids lacking the accessory *Tu*, display the same activity of *c-src* kinase (about 300 cpm/mg protein). This activity appears to be the basic expression of *c-src.* In contrast, the melanoma-bearing hybrids which contain the derepressed *Tu* show an increase in *c-src* activity, with the malignant melanoma bearing BC-hybrids displaying the highest activities (about 600 cpm/mg protein).

In the experiment illustrated in figure 4, all purebred and hybrid animals, irrespective of the lack and the dosage of the accessory *Tu*, but dependent on the nontumorous state exerted either by several regulatory genes or by a linked *R* alone (*see* the highly susceptible genotype), display a uniform *c-src* activity (about 200 cpm/mg protein) which seems to represent the basic *c-src* expression like that in purebred animals and *Tu*-lacking hybrids in the previous experiment (fig. 3).

In littermates (fig. 6) genetically identical except for the lack of the accessory *Tu* and the presence of 1 or 2 partially derepressed accessory *Tu* copies, *c-src* displays a kinase activity that increases stepwise and parallel to the lack and the dosage of *Tu* (100, 200, 400 cpm/mg protein), which, in turn, determine whether the animals will develop no tumors or slow- or rapid-growing tumors. Table 3 shows other experiments of the same kind that yielded similar results.

The main results of these experiments are that the nontumorous fish display a basic expression of *c-src,* which, in the tumorous fish, may increase stepwise under 2 conditions, i.e., the stepwise 1) derepression of an accessory *Tu* and 2) introduction of additional copies of a derepressed accessory *Tu.* These findings indicate several possibilities for an interpretation about how *Tu* is related to *c-src:* 1) It is independent from *c-src,* and the correspondence between both *Tu* and *c-src* is due to linkage relationships. 2) The *c-src* represents a regulatory gene for *Tu* or *vice versa.* 3) It might consist of different oncogenes responsible for different kinds of neoplasia, and *c-src* is one of these genes. 4) Also, *Tu* is identical to *c-src*, and this oncogene can code for a wide variety of neoplasia. At present, we cannot make a firm interpretation; additional data are required.

TABLE 3.—*The pp60^{c-src}-associated kinase activity in brain extracts specified by counts per milligram soluble protein in F_2 segregants*[a]

Tu gene complex[b]	Dosage of *Tu*, cpm/mg[c]		
	None	Single	Double
Striped[d]	90	200	390
Dabbed[e]	170	NT	390
Dabbed[f]	200	260	1,240

[a] Three to 8 brains per measurement were used.

[b] Different gels each were measured.

[c] One gel each was measured. NT = not tested.

[d] The *Tu* copy of *striped* originates from the X- and Y-chromosomes of *X. maculatus* from Rio Jamapa, Mexico; the linked *R* and R_{Bs} are impaired (*see* fig. 4), which resulted in the phenotype shown in figure 6.

[e] The *Tu* copy of *dabbed* originates from an autosome of *X. helleri* from Belize River, British Honduras; *Tu*-control is partly impaired in the compartment of the side of the body.

[f] Phenotype and genotype are similar to those of *e*, but *X. helleri* originates from Rio Lancetilla, Mexico.

DISTRIBUTION OF *c-src* IN THE ANIMAL KINGDOM

The presence of *c-src* in the genome of taxonomically different animals, such as chicken, salmon, and *Xiphophorus,* led us to a more systematic search for this oncogene in additional taxonomic groups of animals. First, different groups of *Xiphophorus* and different fish genera taxonomically related to *Xiphophorus* were investigated. All fish tested (table 4) show a pp60^{c-src} kinase activity indicating that *c-src* must be present (*73*). In addition, *c-src* was evidenced by its kinase activity in a large variety of fish

TABLE 4.—*Expression of pp60^{c-src} kinase in brain extracts of different fish species*[a]

Species	Source
X. helleri	Belize River, British Honduras
X. helleri	Rio Lancetilla, Mexico
X. maculatus	Belize River, British Honduras
"	Rio Jamapa, Mexico
"	Rio Usumacinta, Mexico; British Honduras
X. cortezi	Rio Axtla/Panuco System, Mexico
X. variatus	Rio Coy, Mexico
X. variatus	Rio Panuco, Mexico
Girardinus falcatus	Cuba
G. metallicus	Cuba
Poecilia sphenops	Puerto Barrios, Guatemala
Belonesox belizanus	Chetumal, Mexico
Heterandria bimaculata	Lake Catemaco, Mexico
Xenotoca eiseni	Highlands of Mexico

[a] *See* (*73*).

TABLE 5.—*The* c-src *in eukaryotes*[a]

- Mammals
  - Humans (*70*)
  - Calf (*70*)
  - Rat
  - Mouse
- Birds
  - Chicken
  - Quail
- Bony fish
  - Flat fish
  - Sea robin
  - Mackerel
  - Roach
  - Gudgeon
  - *Xiphophorus*
  - Salmon (*70*)
  - Codfish
  - Cichlid
- Cartilaginous fish
  - Shark
- Jawless fish
  - Lamprey
- Acrania
  - *Amphioxus*
- Insects
  - Cockroach
  - *Drosophila melanogaster* (*78*)
- Sponges
  - Marine sponge
  - Freshwater sponge

[a] *See* (*76*).

and metazoa (other than fish) ranging from mammals to sponges (*76*), which, with the results of other laboratories, are listed in table 5. Schartl and Barnekow (*76*) found no *c-src* in protozoa, algae, and higher plants.

The ubiquity of *c-src* in metazoa suggested that this homolog of the viral oncogene *v-src* still has unknown basic functions of life closely related to the evolution of the multicellular organization of the animals and that neoplasia is a characteristic closely related to this evolution. As the sponges are known to have evolved in the Proterozoic era, the origin of the *c-src* oncogene has to be estimated as having occurred over 1.5×10^9 years ago (*76*).

CONCLUSIONS

We studied neoplasia of *Xiphophorus* at different levels of the biological organization, including species, races, populations, generations, littermates, embryos, adults, tissues, cells, genomes, chromosomes, and genes and gene products. In doing so, we traced neoplastic transformation to the activity of 1 copy or several copies of the oncogene *Tu* that shows a relation to the cellular counterpart of the transforming oncogene of Rous sarcoma virus, the *c-src*.

Although we cannot decide now what the basis of the relationship between the Mendelian *Tu* and the molecular *c-src* in *Xiphophorus* might be (linkage, identity, etc.), and, although we still do not have data on the distribution of *Tu* in the animal kingdom comparable to that of *c-src*, our experiments support the assumption that the biological fundamentals of neoplasia are, in principle, the same in all groups of metazoa including humans (*77*). Genes that are apparently essential for the differentiation and proliferation of cells to form tissues in the early embryo and to maintain the differentiated state of the tissue by cell-cell communication in development and during adulthood obviously can relapse into their early function, which, beyond the early embryonic state, appears as the transforming function of the oncogene.

Of the acutely transforming retroviruses thus far investigated, all have oncogenes related to cellular genes that are also present in the normal genome of various vertebrate species. We need to know whether they are also present in *Xiphophorus* and, if so, whether their function is also related to *Tu*. No other animal model would be as suitable for this research as the *Xiphophorus* fish model.

Normally, the oncogenes are under the control of tissue-specific systems of regulatory genes that have evolved population specifically. Interpopulational and interracial hybridization in the preceding generation is the main event contributing to the disintegration of the regulatory gene systems for the oncogene. Germ-line mutations that may also disturb the regulatory gene systems are probably less important than hybridization because they are always rare or may become repaired. Somatic mutations, which are among the major factors that trigger carcinogenicity, may complete this disintegration. This implies that somatic mutation is the primary end of a sequence of events that leads to initiation of neoplasia (*4, 5, 35, 77*).

Although we do not have data on interpopulational hybridization in human beings comparable to those of *Xiphophorus* or to the domestic and the classical laboratory animals, it is interesting for one to speculate how much effect hybridization might have contributed to the sequence of events that led to the high tumor incidence observable in most of our highly developed nations. Such speculations are probably of little value for our fight against cancer in humans but they could help to identify the factors that make an individual insensitive or sensitive to the carcinogens of our environment. They could further help in our decisions whether we should direct our attention more to the promoters in our environment or to the initiators because elimination of initiators would have less effect in our fight against cancer if individuals would have already undergone important events of initiation.

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Use of the Mudminnow, *Umbra* sp., in an In Vivo Sister Chromatid Exchange Test[1]

Andrew D. Kligerman,[2] William E. Bishop,[3] *and* Laura C. Valentine[3]

ABSTRACT—Elevated frequencies of sister chromatid exchange (SCE) have been observed in the chromosomes of mammals and birds as a result of exposure to mutagenic carcinogens. More recently, this phenomenon has been demonstrated in the chromosomes of fishes exposed to genotoxicants. A short-term screening system for mutagenic carcinogens with the use of the mudminnow, *Umbra* sp., has been developed with SCE as the indicator of genetic damage. This genus of fish possesses a small number ($2n=22$) of large chromosomes enabling easy and rapid quantitation of cytogenetic damage. When mudminnows were exposed by ip injection or in situ by the aquatic environment to either direct- or indirect-acting mutagens, dose-dependent increases in SCE were observed. In addition to screening capabilities, the mudminnow system has had application as a biological monitor for genotoxicants in contaminated water systems. The increasing use of the mudminnow SCE system illustrates a renewed interest in the utilization of fishes in mutagenicity and carcinogenicity testing. — Natl Cancer Inst Monogr 65: 111–118, 1984.

The analysis of SCE has been used in various in vivo test systems (*1–4*) as an indicator of genetic damage resulting from exposure to genotoxic agents. The SCE involves the apparently reciprocal exchange between portions of DNA double helices and may reflect the ability of a cell to replicate DNA on a damaged template and continue the process of cell division (*5–7*). The precise mechanism of SCE induction has not been elucidated, but the association between SCE and exposure to mutagenic substances is strong (*8–11*). Because SCE can be induced by low concentrations of mutagenic carcinogens (*8, 12*), evaluation of SCE frequency provides a sensitive and rapid method for the assessment of genetic damage. Thus the incorporation of the SCE methodology into an aquatic test system would be well-suited for the study of waterborne genotoxicants.

Originally, Kligerman et al. (*13*) developed a cytogenetic system for the study of chromosome breakage in fishes using the mudminnow, *Umbra limi,* as a model organism. After the development of the in vitro BrdUrd-mediated SCE methodology by Latt (*14*), Bloom (*1*) suggested that the visualization of SCE in vivo would be possible with the mudminnow, as he had recently shown in ovo with the chick embryo. This led to the development of the SCE system, with *U. limi,* the central mudminnow, in the United States, and *U. pygmaea,* the eastern mudminnow, in Europe.

As a short-term in vivo test, the mudminnow system can be applied as a screen for the detection of mutagenic compounds and as a biological indicator of waterborne mutagens in polluted waters. Fishes have many advantages for such studies. They are aquatic vertebrates that have the ability to concentrate low levels of xenobiotics from the surrounding environment. Also, they possess the enzymatic capabilities necessary for the conversion of promutagens and procarcinogens to their ultimate reactive metabolites (*15, 16*). Because these studies can be done in vivo, the complex problems of activation, transport, and excretion are accounted for inherently. Furthermore, various tissues can be examined in the same animal, which may aid investigators in detecting the action of site-specific agents.

THE TEST ORGANISM

The mudminnow is a small (5 to 10 cm), olive brown-colored teleost that inhabits slow-moving or stagnant mud-bottomed streams and ponds. Only 3 species of the genus are known: *U. limi,* found in central and northeastern North America, and *U. pygmaea,* which inhabits lowland freshwaters along the Atlantic coast (*17*), have been used in cytogenetic research. (The central European species, *U. krameri,* has not been the subject of cytogenetic investigations.) The economic significance of Umbridae exists in its value as bait, and it has been suggested as a biological control organism for reducing mosquito populations (*18*).

The major factor facilitating the use of mudminnows in cytogenetic investigations is the karyotype. Unlike most teleosts which have large numbers ($2n \geqslant 48$) of small chromosomes ($<3\ \mu$m), *Umbra* have only 22 large (3–8 μm) metacentric and submetacentric chromosomes (fig. 1). Its innate tolerance of adverse environmental conditions enables the mudminnow to withstand the stresses of experimental conditions, which often involve ip injections of toxins.

At present, the mudminnow is not commercially available. However, they can be seined from mud-bottomed ponds and slow-moving streams throughout most of their

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ABBREVIATIONS: SCE = sister chromatid exchange(s); BrdUrd = 5-bromodeoxyuridine; R = roentgens.

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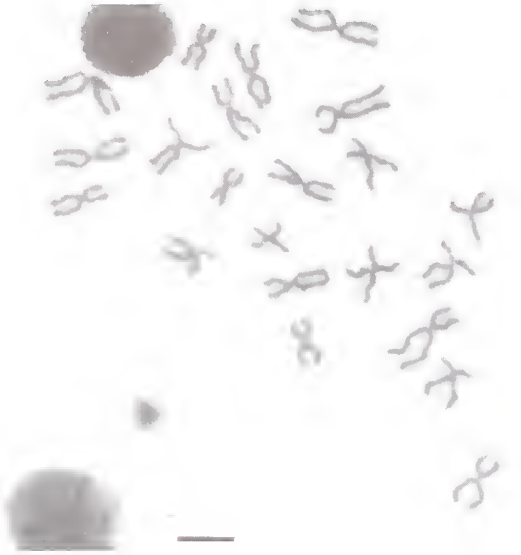

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FIGURE 1.—Metaphase from the intestines of *U. limi* (2n=22). Bar=5 μm.

range. Although they are difficult to breed in laboratory aquaria, they will reproduce in outdoor shallow mud-bottomed ponds. Once the pond is stocked, fish are readily available for laboratory research.

Fish can be maintained in the laboratory in filtered, well-aerated aquaria. The water hardness and pH should approach that of the body of water from which they were taken, and overcrowding should be avoided. Mudminnows can tolerate a wide range of temperatures (0° to 30° C), but the temperature should remain stable. Because fish are poikilothermic, their mitotic activity and food consumption will vary with water temperature. For cytogenetic studies, temperatures between 20° and 25° C are recommended, but maintenance temperatures of 15° to 20° C are adequate. Because they do not readily accept flaked or dry food, feeding the fish in the laboratory has been a problem for researchers. Aquatic invertebrates make up a large part of their diet in the wild and, if available, are the best source of food for animals in the laboratory aquaria. In some laboratories, fish have been weaned of live food and on to freeze-dried or pelleted foods. Care should be taken that the food is not a source of genotoxic contamination that could cause cytogenetic damage.

When fish are treated with chemical agents for cytogenetic studies, it is best for the investigator to use all glass aquaria with no gravel or plants. This permits easy cleaning and disposal of contaminated water and also limits accumulation or biotransformation of text chemicals by other organisms. Either stationary or flow-through systems can be constructed, depending on the aims of the experimenters. The water should be well aerated, but one should take care to prevent the escape of aerosols containing potentially dangerous compounds. For adequate mitotic activity, fish should be fed during exposures. Any food not consumed should be removed so that the water is not fouled.

TEST DEVELOPMENT

Kligerman et al. (*13*) demonstrated the mudminnow's utility as an in vivo cytogenetics aquatic model with experiments involving whole body X-irradiation. Mudminnows exposed to 325 R showed chromatid breaks and gaps in almost 30% of the metaphases examined. These findings were confirmed and expanded upon by Mong and Berra (*19*) who generated a dose-response curve for mudminnows exposed to X-rays.

Because SCE analysis is more sensitive than chromosome breakage analysis for detection of the presence of genotoxicants (*8*), the BrdUrd methodology (*14*) for visualizing SCE was incorporated into the system (*20-22*). The basis of this technique is that if cells are allowed to replicate in the presence of BrdUrd for two rounds of DNA synthesis, BrdUrd will replace the thymidine in one strand of the DNA helix of one chromatid and in both strands of the helix in its sister chromatid (fig. 2). Sister chromatid differentiation will result because the doubly substituted strand will either fluoresce less intensely after staining with 33258 Hoechst (fig. 3A) or stain less strongly following a fluorochrome plus Giemsa treatment (figs. 3B, C) than the singly substituted strand. The resultant sister chromatid differentiation permits the clear visualization of SCE.

The mudminnow does not metabolize BrdUrd rapidly. Kligerman and Bloom (*20*) found that after a single injection of 500 μg BrdUrd/g into the abdominal cavity of the mudminnow, sister chromatid differentiation could be detected in metaphases from the gills, kidneys, intestines, and scale epithelium. They investigated several variables to try to maximize the percentage of metaphases that would display sister chromatid differentiation. After a single injection of 500 μg BrdUrd/g, the intestines showed the greatest number of cells with sister chromatid differentiation, followed by metaphases from the kidneys, gills, and scales. No consistent dose-related increases in the frequency of differentially stained metaphases were observed as the amount of BrdUrd injected increased from 250 to 1,000 μg/g, but the clarity of differentiation was improved at doses of 500 μg/g or more. Variation in

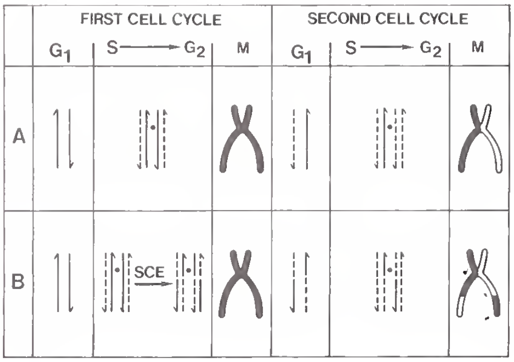

FIGURE 2.—Schematic representation of BrdUrd incorporation leading to a sister chromatid differentiation. A) No SCE occur. B) One SCE occurs. Figure is reproduced with permission of the publisher (*23*).

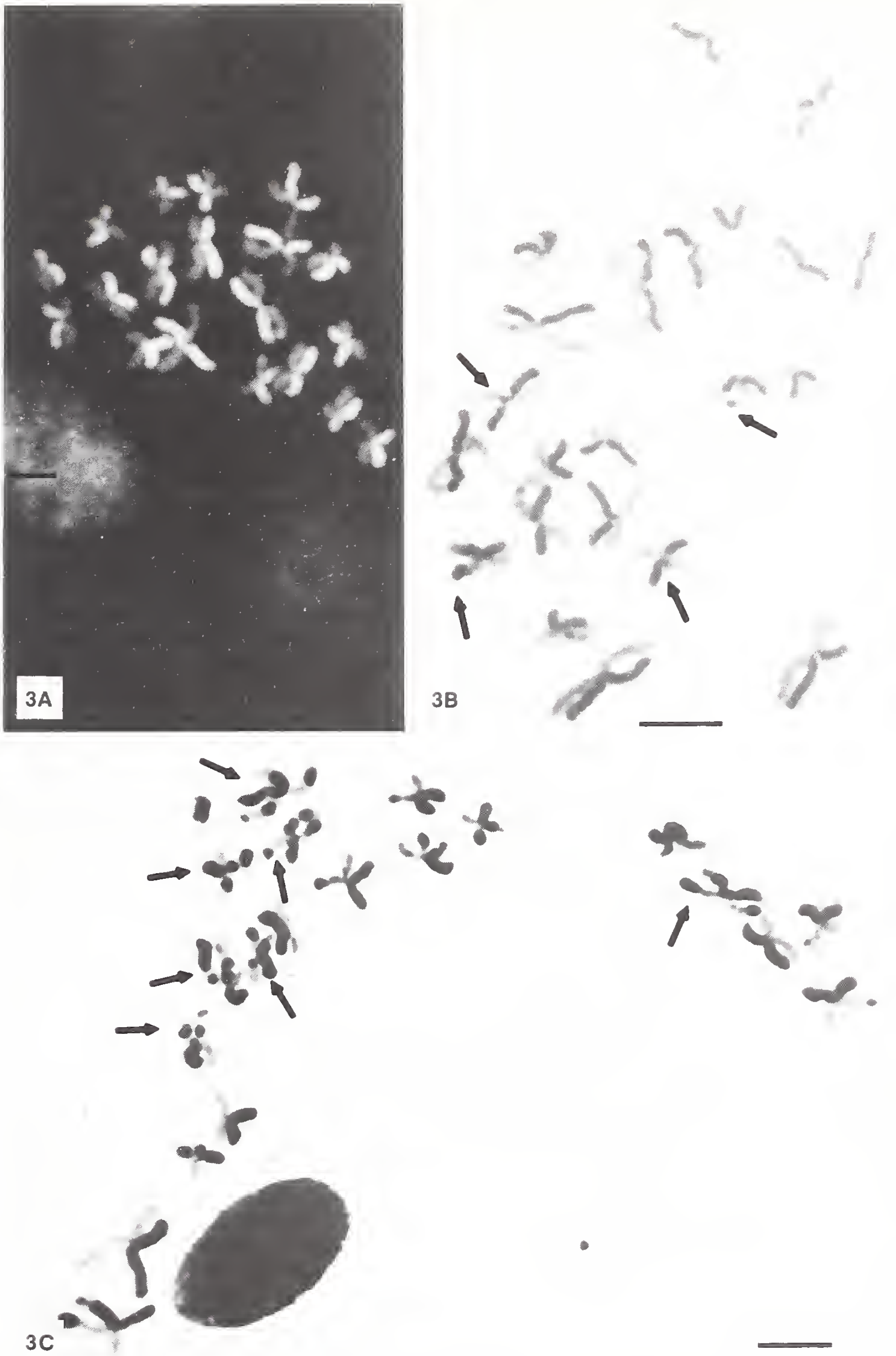

FIGURE 3.—A) Hoechst-stained metaphase from *U. limi*. B) Fluorescence plus Giemsa-stained metaphase from *U. limi*. *Arrows* denote SCE. C) Metaphase from *U. limi* treated with 66 μg methyl methanesulfonate/g. *Arrows* denote some of the chromosomes with multiple SCE. Bar = 5 μm.

duration of exposure of the fishes to BrdUrd showed that the peak number of metaphases with sister chromatid differentiation occurred between 5 and 6.5 days after the injection (*21*). Thus the cell cycle time for the mudminnow at 18° C ranges from about 2½ to 3½ days depending on the cell type examined.[4] The base-line SCE frequency was low, averaging about 2.6, 3.1, and 4.1 for the gills, kidneys, and intestines, respectively (*22*). Since the initial investigations, other researchers have found similar control SCE frequencies (table 1).

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[4] Cell cycle time will vary with temperature and possibly nutritional status of fishes. This parameter should be determined by investigators in their laboratories by using sequential times for killing the fish after administration of BrdUrd.

TABLE 1.—*Control SCE frequencies from selected tissues and species*

Organism	Tissue	SCE/metaphase	Reference
U. limi	Gills	2.4	(*20*)
	Intestines	2.6	
U. limi	Gills	2.6	(*22*)
	Intestines	4.1	
	Kidneys	3.1	
U. limi	Intestines	2.7	(*23*)
U. pygmaea	Gills	3.1[a]	(*24*)
	Testes	3.1	
U. pygmaea	Gills	2.7	(*25*)
	Fins	2.2	

[a] The value was calculated from average SCE/chromosome data presented by the authors of (*24*).

TEST METHOD

Fish are given ip injections of 500 μg BrdUrd/g, and 24 hours later,[5] the fish are exposed to the test agent by an ip injection or the addition of the agent to the aquarium water. Four days later and approximately 7 hours before they are killed, we gave each fish an ip injection of 25 μg Colcemid/g or colchicine to block dividing cells at metaphase. Tissues are prepared by the solid tissue technique (*26*). Briefly, the tissues are removed from the fish and treated with a hypotonic solution (0.4% potassium chloride) for 30 minutes. They are fixed overnight in methanol:acetic acid and macerated in 50% acetic acid in the well of a depression slide to form a dilute cell suspension. The cell suspension is transferred to a heated slide (50° C) to form a ring-shaped monolayer of cells. Slides are stained by a modification of a fluorescence-plus-Giemsa technique (*27*), which entails a 10-minute treatment with 33258 Hoechst (200 μg/ml) followed by exposure of wet mounted slides (McIlvaine's buffer, pH 7.0) to intense fluorescent lights for 30–45 minutes. Then slides are stained in 3% Giemsa (pH 7.0), air dried, cleared with xylol, and mounted. Metaphase spreads are examined under oil immersion (× 1,125) and scored for SCE (fig. 4).

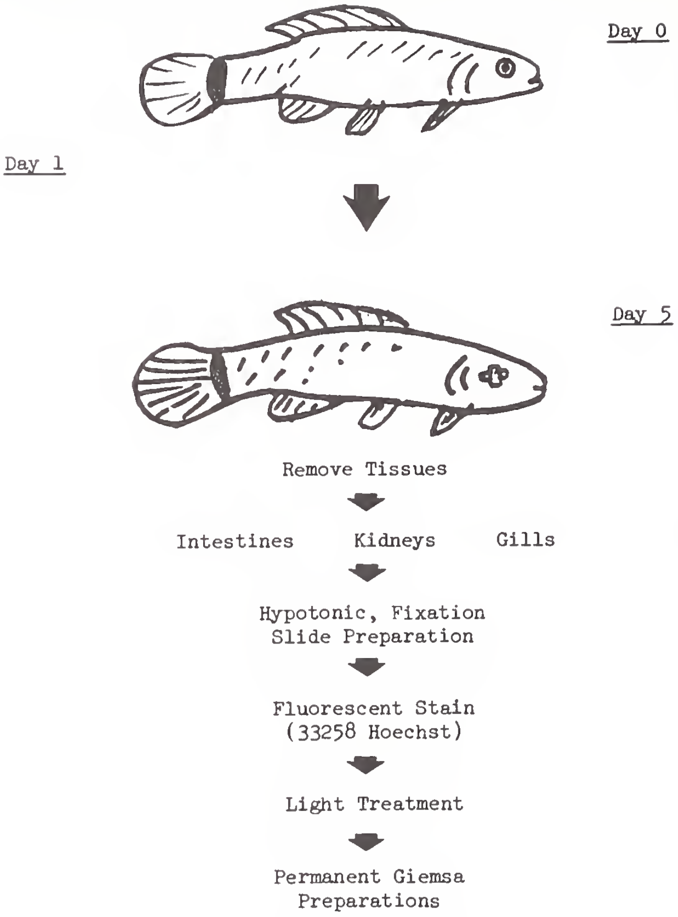

FIGURE 4.—Pictorial representation of the experimental protocol for visualizing SCE in metaphases from *U. limi.* Day 0: BrdUrd injection; day 1: mutagen treatment; day 5: Colcemid injection, 7-hr incubation, and fish killed. Figure is reproduced with permission of the publisher (*22*).

APPLICATIONS OF THE MUDMINNOW SYSTEM

The initial studies with the mudminnow system were performed for determination of whether SCE could be induced by exposure of the fish to direct- and indirect-acting mutagens (methyl methanesulfonate and cyclophosphamide, respectively) by ip injection, and by the addition of an indirect-acting mutagen [neutral red dye (3-amino-7-dimethyl-amino-2-methylphenazine·hydrochloric acid)] to the aquarium water (table 2). For each tissue examined (gills, kidneys, and intestines) and over the concentration range used, a significant dose–response was found (*22*), as illustrated in figure 5. These experiments demonstrated that 1) SCE can be induced in the mudminnow after ip or in situ exposure to mutagens, 2) the SCE increase occurs in a dose-dependent fashion, and 3) the mudminnow is capable of metabolizing at least some promutagens to ultimate genotoxic metabolites.

Recently, Bishop and Valentine (*23*) confirmed these findings for methyl methanesulfonate and cyclophosphamide and extended the data base to include the direct-acting mutagen, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, and the promutagen, dimethylnitrosamine. A dose-dependent increase in SCE frequency was found for all 4 compounds. By comparing the slopes of the equations describing the approximately linear curves, they ranked the 4 compounds according to their SCE-inducing potential (dimethylnitrosamine <methyl methanesulfonate <cyclophosphamide <*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine).

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[5] The test agent can be given either before, concurrent, or after the BrdUrd injection. The only restrictions are that the BrdUrd must be given 2 DNA replications before fish are killed and the test agent administered sometime before the final replication period before killing. For field studies, it is recommended that the BrdUrd be injected after exposure, so that the length of the field study would not be restricted by the cell cycle time.

TABLE 2.—*Induction of SCE and chromosome breakage in the mudminnow by genotoxicants*

Analyses	Species	Exposure	Amount	Route	Tissue	Response[a]	Reference
SCE	*U. limi*	Methyl methanesulfonate	0–103 μg/g	ip	Gills	++	(*22*)
					Kidneys	++	
					Intestines	++	
		Cyclophosphamide	0–22 μg/g	ip	Gills	++	
					Kidneys	++	
					Intestines	++	
		Neutral red dye	0–12.5 μg/g	in situ[b]	Gills	++	
					Kidneys	+	
					Intestines	++	
	U. limi	Methyl methanesulfonate	0–250 μg/g	ip	Intestines	++	(*23*)
		Cyclophosphamide	0–300 μg/g	ip	Intestines	++	
		Dimethylnitrosamine	0–2,000 μg/g	ip	Intestines	++	
		N-methyl-*N*′-nitro-*N*-nitrosoguanidine	0–30 μg/g	ip	Intestines	++	
Chromosome breakage	*U. limi*	X-ray	0, 325 R	in situ[c]	Gills	++	(*13*)
					Kidneys		
					Intestines		
	U. limi	X-ray	0–990 R	in situ[c]	Gills	++	(*19*)
					Spleen		
	U. pygmaea	Ethyl methanesulfonate	0–200 mg/liter	in situ[b]	Gills	++	(*25*)
					Fins	++	
		Benzo[*a*]pyrene	0–0.1 μg/liter		Gills	++	
					Fins	++	
	U. pygmaea	Trenimon	0–20 μg/liter	in situ[b]	Fins	++	(*30*)
					Scales		
					Intestines		

[a] + = positive response with at least a 50% increase over control at the highest concentration examined; ++ = positive response with at least a 100% increase over control.

[b] The chemical was added to the aquarium water.

[c] Fish were irradiated in a small volume of water (100–300 ml).

In Europe, investigators adapted the techniques used with *U. limi* to investigate mutagenic effects caused by exposure of the eastern mudminnow, *U. pygmaea,* to mutagenic carcinogens and polluted water. Hooftman and Vink (*25*) added 8 mg ethyl methanesulfonate/liter or 0.1 μg benzo[*a*]pyrene/liter aquarium water; after 48 hours significant increases in chromosome breakage were observed. Hooftman and Vink (*25*) exposed mudminnows to water from the Rhine River for 8 days and observed an approximate doubling in SCE frequency and chromosome aberrations in metaphases from the gills. In metaphases from fin tissue, they found a threefold to fourfold increase in SCE frequency over controls. Similarly, Alink et al. (*24*) and Prein and co-workers (*29*) exposed mudminnows to water from the same river for 3 to 11 days. The latter group found almost a fourfold increase in the number of chromosome aberrations in the gills, and Alink et al. (*24*) reported that metaphases from the gills and testes exhibited a twofold to threefold increase in SCE frequencies. However, Sugatt (*30*) detected no significant increase in chromosome breakage in mudminnows exposed to polluted water from the Lek and Mass rivers in The Netherlands. He did find that the addition of 10 to 20 μg Trenimon/liter (2,3,5-trisethyleneimino-1,4-benzoquinone) to the aquaria caused a significant increase in chromosome breakage and had a definite cytostatic effect.

Thus the mudminnow system has shown promise for use as an aquatic model for the cytogenetic detection of genotoxicants. However, more work is necessary if the system is to be validated. Animals should be exposed to genotoxic as well as nongenotoxic analogs and polluted as well as nonpolluted water sources for determination of the sensitivity and selectivity of the assay.

DISCUSSION

The incorporation of both chromosome breakage and SCE analyses in an aquatic system for the detection of mutagens offers many advantages. Cytogenetic preparations give actual visual records of the effects of genotoxic agents on the chromatin. Thus there is little doubt that the agent under study has the ability to interact with the genetic material if a positive response is obtained. Usually, SCE analysis is the more sensitive method, as one can detect exposure to mutagenic carcinogens at concentrations sometimes orders of magnitude lower than those detected by the analysis of chromosome breakage (*8*). However, the use of chromosome breakage is important if one is to detect the effects of ionizing radiation and those small numbers of chemicals [e.g., bleomycin (*8*)] that produce chromosome breakage without causing significant elevations in SCE frequency. In both instances, the methodology is simple and rapid, and statistically significant results can be obtained with small sample sizes.

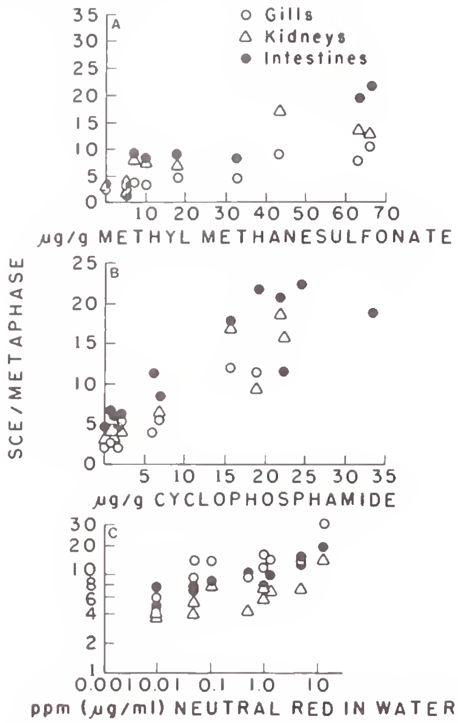

FIGURE 5.—Effects of mutagen administration on the SCE frequencies of the gills, intestines, and kidneys of central mudminnows. Fish were given injections of methyl methanesulfonate (A), cyclophosphamide (B), or exposed to neutral red dye (C) in the aquarium water. The 3 tissue values at each concentration point were obtained from 1 fish. Figure is reproduced with permission of the publisher (*28*).

The use of the mudminnow as an aquatic model for both field and laboratory studies is superior to in vitro, prokaryote, or lower eukaryote test systems because relevant exposure conditions can be examined. Factors such as bioconcentration, metabolism, blood–tissue barriers, differential tissue sensitivity, dilution, and excretion are taken into account. Studies can be conducted in which water samples are brought into the laboratory. Then the fishes could be exposed to the water samples or concentrates prepared from the samples. Also, one could envision studies in which mudminnows are caged in water bodies for various lengths of time and then returned to the laboratory for cytogenetic analyses.

The potential for use of aquatic organisms for the detection of cytogenetic damage caused by environmental pollutants should not be limited to the species or system described here. The major restriction on the choice of species used for cytogenetic studies is the karyotype. This should consist of a small number of large chromosomes (*28, 31*). The organism should also be easily and inexpensively maintained. Other organisms, such as the oyster toadfish, *Opsanus tau* (*32*), or the marine polycheate, *Neanthes arenaceodentata* (*33*), have already shown promise for marine and benthic assays. Recently, *Notobranchius rachowi*, a freshwater tropical fish, has compared favorably to the mudminnow in its sensitivity to mutagens (*34*) and the ease with which cytogenetic damage can be scored.

Another method that has been underutilized is the culture of lymphocytes from exposed fishes. Lymphocyte culture involves the removal of a small amount of blood from control and exposed animals and the stimulation of the blood to divide with mitogens, such as phytohemagglu-

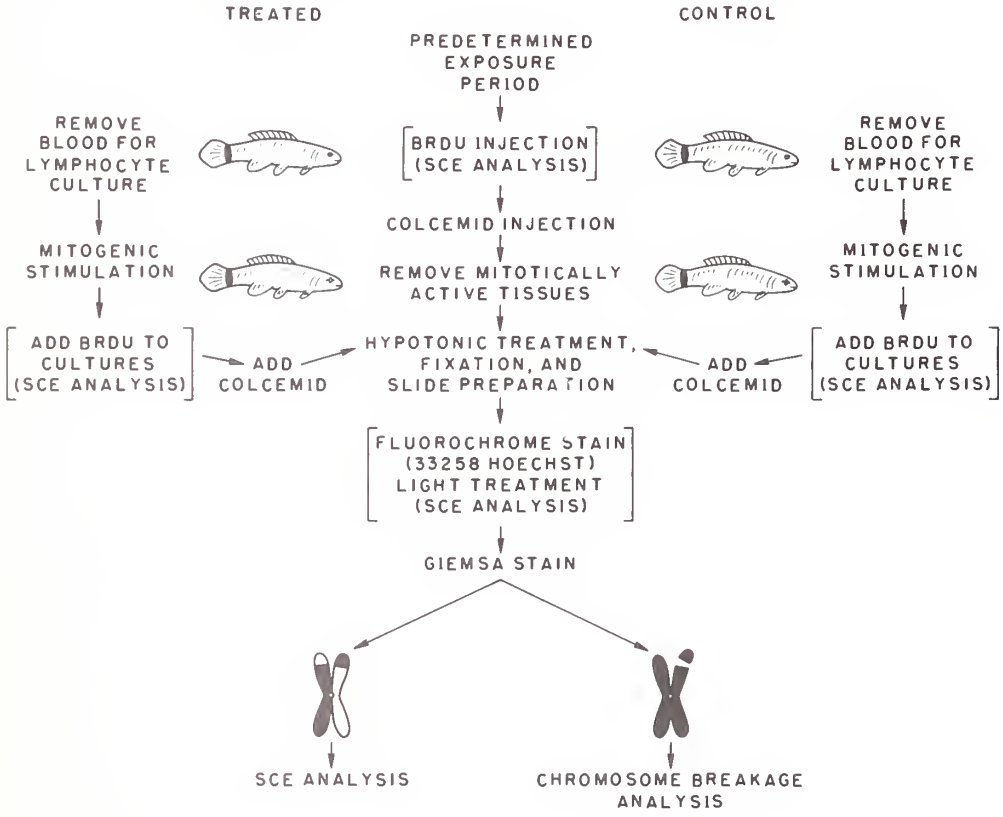
TREATED
CONTROL
PREDETERMINED EXPOSURE PERIOD
REMOVE BLOOD FOR LYMPHOCYTE CULTURE
BRDU INJECTION (SCE ANALYSIS)
REMOVE BLOOD FOR LYMPHOCYTE CULTURE
COLCEMID INJECTION
MITOGENIC STIMULATION
REMOVE MITOTICALLY ACTIVE TISSUES
MITOGENIC STIMULATION
ADD BRDU TO CULTURES (SCE ANALYSIS)
ADD COLCEMID
HYPOTONIC TREATMENT, FIXATION, AND SLIDE PREPARATION
ADD COLCEMID
ADD BRDU TO CULTURES (SCE ANALYSIS)
FLUOROCHROME STAIN (33258 HOECHST) LIGHT TREATMENT (SCE ANALYSIS)
GIEMSA STAIN
SCE ANALYSIS
CHROMOSOME BREAKAGE ANALYSIS

FIGURE 6.—Proposed methodology for study of chromosome breakage and SCE in solid tissues and lymphocyte cultures from fishes. BRDU = 5-bromodeoxyuridine. Figure is reproduced with permission of the publisher (*28*).

tinin, pokeweed, and concanavalin A. Then BrdUrd is added to the cultures and the SCE frequency measured after the cells have replicated twice. If the animal is large enough, the blood can be removed nonlethally, and cytogenetic damage could be analyzed before and after exposure in the same animal. The major problem that must be overcome is the determination of appropriate medium, mitogen, and culture conditions for lymphocyte division. If lymphocyte culture were successful, the problem of low mitotic indexes found in most fishes could be overcome. Some promising attempts have been made by Maddock and Kelly (*32*) and Stromberg et al. (*35*), but lymphocyte culture has not been utilized for fishes exposed in vivo. One can envision a protocol similar to that shown in figure 6 in which both SCE and chromosome breakage can be studied in the same animals using the solid tissue technique or lymphocyte culture to obtain metaphase preparations.

SUMMARY

The mudminnows, *Umbra* sp., have been shown to be useful test organisms for the detection of potentially genotoxic substances in both the aquatic environment and in a laboratory screening program. Investigators should continue aquatic genetic toxicology research to improve and validate these and similar systems, to test their utility in field studies, and to increase the data base of compounds that have been examined.

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DISCUSSION

K. Hoover: I just wonder, do you think that temperature would play any kind of role in the frequency with which you would see SCE?

A. D. Kligerman: Little, if any. I can remember 1 study in which, by denaturing some enzymes in tissue culture, you can slightly affect the SCE frequency, but temperature has little effect on an SCE frequency. I really cannot think of an area where it would. Maybe an increase of 1 SCE would be likely, but substantial increases would seem unlikely.

Hoover: I guess it might play a role in metabolism.

Kligerman: Yes, it could do that. Also, it could act in some of the other functions of transport and similar activities, but I think it would not greatly affect the baseline SCE frequency. It might effect the incorporation of the BrdUrd.

G. Streisinger: Have you looked at compounds with a low Swainscott factor, such as ethylnitrosourea? Compounds with low Swainscott factors are highly specific with respect to the places at which they alkylate.

I wondered whether those agents, ethylnitrosourea is a good example, also give hefty SCE.

Kligerman: Ethylnitrosourea does induce SCE. All the compounds I looked at are shown in the table.

J. Bend: One of the active areas of research now, certainly in relationships between chemical metabolism and toxicity, is the role of cellular heterogeneity. A number of the tissues in which you are showing your SCE are, in fact, heterogeneous cell types.

Is there any potential with your system to detect those cells that would be high in monooxidase activity and to look specifically in those cells for exchange?

Kligerman: It is hard to say. I would doubt it to the extent that usually the cells that are dividing are the undifferentiated cells, and they are generally of 1 type. You will not find highly differentiated cells dividing. So, I would say no in that case, but because you are looking at different tissue types, there is some potential to compare the differences in tissues.

K. Kator: Do only mutagens or carcinogens enhance SCE? I think not.

Kligerman: For the most part, yes, some compounds are not mutagenic nor carcinogenic yet do induce SCE, as we know, but those are in the minority. It might be found that they really are carcinogenic, if the studies are done correctly.

P. Weiss: I noticed that in the presentation of the data you presented SCE/cell, but you also talked about SCE/chromosome, as well. I was wondering how you scored these.

Kligerman: My data were always presented "per cell." Some people presented "per chromosome." I guess there is no problem if you are counting all the chromosomes in every cell.

People do present SCE per chromosome a lot of times because they find incomplete cells, and they do not want to throw them out, so they just total the number of SCE and divide by the number of chromosomes. If the chromosomes in the cell are all similar, you have no problem, but if they are not, it might lead to a problem.

Weiss: However, is it possible to get a high score even if all the breaks are only in 1 chromosomal arm?

Kligerman: You almost never find them isolated in 1 chromosome. They are randomly distributed, for the most part, throughout the cells and throughout the chromosomes.

Hyperplastic Lesions of the Primitive Meninx of Fathead Minnows, *Pimephales promelas,* Induced by Ammonia: Species Potential for Carcinogen Testing[1, 2]

Charlie E. Smith[3, 4]

ABSTRACT—In our laboratory, fathead minnows were reared from time of hatching to adulthood at 5 concentrations of ammonia in a toxicity test lasting 304 days. The ammonia concentration test range was 9.36 to 30.0 mg NH_3-N/liter (0.639 to 2.17 mg NH_3/liter). Intracerebral lesions were observed in fish from each ammonia concentration but not in the controls. The severity of the lesions was positively correlated with increased ammonia concentration. Massive hyperplastic lesions developed in fish tested at the 2 highest concentrations. Histologically, lesions appeared to originate from the primitive meninx, which grew into the cranial cavity, and in some fish through the cranium. Based on the orderly appearance of tissues and evidence of regression of lesions, the lesions are not considered to be neoplasms but hyperplasias of primitive meninx tissue induced as a response to ammonia exposure. Fathead minnows (*Pimephales promelas*) have proved useful for acute toxicity testing of environmental contaminants and have a potential usefulness in carcinogenicity testing. They are easily maintained in the laboratory, can be readily induced to spawn during their first year of life, and have a relatively short period of embryogenesis. — Natl Cancer Inst Monogr 65: 119–125, 1984.

GEOGRAPHICAL RANGE

The fathead minnow (*Pimephales promelas,* family Cyprinidae) is probably the most easily propagated minnow indigenous to a geographical range including southern Canada, the United States, and northern Mexico. Viable populations are present from sea level to an elevation of 3,000 m (*1*). The species is widely cultured for bait and forage fish and is used extensively at various laboratories throughout the United States (*2*) as an assay fish for the determination of toxicity of environmental pollutants.

APPEARANCE

Fathead minnows are typical of cyprinids in appearance and average 50–70 mm in length, with a maximum length of about 100 mm. Prior to spawning, fish exhibit sexual dimorphism; the male develops a generally black color with vertical bands of golden brown, a swollen blunt head with breeding tubercles (pearl organs), and a thickened rugose nape pad resulting from increases in epidermal and dermal thickness and mucous cell number (*3*), as illustrated in figure 1. The female is silvery to olivaceous in color, has a more pointed head than the male, and, when gravid, has a definite distended abdomen. The surest and easiest way one can recognize sexes is the presence of an ovipositor in the female (fig. 2); this can usually be seen by flexing the fish and thereby putting pressure on the abdomen (*4*).

LIFE CYCLE

When exposed to seasonal environmental conditions, the fathead minnow is an intermittent, multiple-spawning fish. Spawning occurs during the summer, generally within the range of 18° to 30° C. Before spawning, the male locates and defends a suitable nesting site beneath floating objects or vegetation; in the laboratory, spawning will occur on the undersurface of spawning tiles. During spawning, eggs are attached to the underside of the nest by an adhesive coating on the chorion. After eggs are deposited and fertilized, the female is driven from the nest by the male (*2*). Embryogenesis takes approximately 5 to 6 days at 25° C. Eggs are about 1.0–1.25 mm in diameter and are laid in clusters, usually 1 egg deep in groups of about 250 to 500. More than 1 female may deposit eggs on the same nest cared for by a single male, and as many as 12,000 eggs/nest have been recorded (*5*).

Both temperature and photoperiod control spawning by fathead minnows. Various minimum temperatures and dates of first spawning have been observed by different investigators, and, although some minimum temperature requirement is indicated, the large number of first spawnings in May strongly support day length as the major influence initiating spawning activity. However, temperature may be more important than photoperiod once the reproductive cycle has been started. Actual first date of spawning is probably determined by complex interrelationships involving temperature, light, and severity of the preceding winter with its effects on the general condition of the fish as spawning approaches and gonads mature (*2*).

ABBREVIATIONS: DMBA = 7,12-dimethylbenz[*a*]anthracene; H & E = hematoxylin and eosin.

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[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Supported in part by grants R803950 and CR807240 from the Environmental Research Laboratory of the United States Environmental Protection Agency, Duluth, Minnesota.

[3] Fish Cultural Development Center, United States Fish and Wildlife Service, Bozeman, Montana 59715.

[4] I thank Mr. John Morrison, Ms. Elizabeth Meyn, and Dr. Robert V. Thurston for their technical assistance.

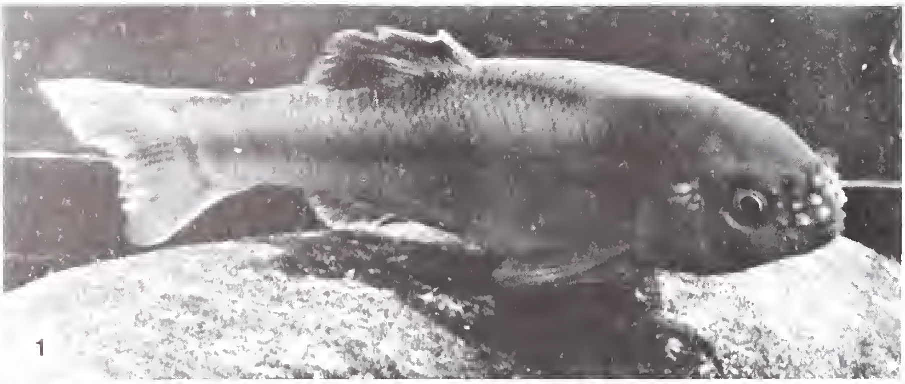


FIGURE 1.—Adult male fathead minnow. *Note* breeding tubercles on snout and thickened pad anterior to dorsal fin. (Figure is used through the courtesy of S. Flickinger.)

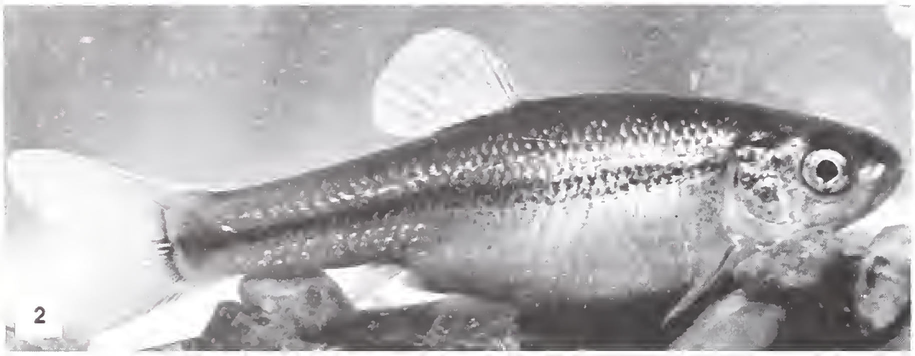


FIGURE 2.—Adult female fathead minnow. *Note* swollen abdomen and the presence of an ovipositor. (Figure is used through the courtesy of S. Flickinger.)

A mature female may be expected to produce between 1,000 and 10,000 eggs from several spawnings over the course of a breeding season, with the total number depending primarily on female size and environmental conditions (*2*).

Under controlled spawning in the laboratory environment, fathead minnow eggs may be obtained throughout the year. This is accomplished with maintenance of optimum temperature and photoperiod (*6*). Till (*7*) described a laboratory technique for obtaining freshly fertilized eggs for use in toxicity experiments. Basic embryological information on the fathead minnow describes development of the embryo from fertilization through the 13-somite stage (*8*) as shown in table 1.

TABLE 1.—*Fathead minnow stages*[a]

Stage No.	Stage name	Time from fertilization, hr[b]
1	Fertilization	0.0
2	2-Cell	1.50
3	4-Cell	2.00
4	8-Cell	2.50
5	16-Cell	2.75
6	Late cleavage	4.00
7	Blastula	5.00
8	Gastrula	7.00–17.00
9	Neurula	16.00
10	Head formation	18.00
11	5-Somite	24.00
12	13-Somite	33.00

[a] Data are reproduced with permission from (*8*).
[b] Temperature at fertilization was 23° C.

REARING REQUIREMENTS

Space may be a limiting factor for the number of fish which can be supported within a contained area. Optimum stocking density of fathead minnow fry in ponds is 100,000–300,000 fish/acre (at 1/4 hectare) or about 0.05 fish/liter (*2, 4*). Smith et al. (*9*) established a tolerance density of 0.17 fish/liter in small 90-liter laboratory tanks. Generally in most chronic toxicity tests, 40–50 fry are placed in 60- to 90-liter aquaria. This number is usually reduced to 15 fish at the end of 60 days and further reduced 6–8 months later at the onset of the spawning period.

In his study on oxygen requirements of fathead minnows, Brungs (*10*) reported that the number of eggs per female was reduced at 2.0 mg/liter, and no spawning occurred at 1.0 mg/liter. Furthermore, fry survival was reduced at 4.0 mg/liter, and approximately one-fifth of those fry that did survive at that concentration were deformed. Recommended oxygen concentrations for fathead minnow toxicity tests is 60% of saturation or greater (*6*). Water temperature and pH have been shown to have a definite influence on reproduction of fathead minnows. Brungs (*11*) exposed fathead minnows continuously to elevated water temperatures ranging from 26° to 34° C. He found that reproduction was more sensitive than was survival, growth, or egg hatchability in assessing the effect of temperature. Growth was reduced and no spawning or mortality occurred at 32° C.

Mount (*12*) maintained fathead minnows continuously for 13 months in water at different pH values from 4.5 to 7.5. At pH 5.2, females became gravid and sexually mature but did not spawn. Egg production and hatchability were reduced at pH 5.9 and lower, and all eggs were abnormal. A pH of 6.6 was marginal for vital functions but safe for continuous exposure.

Standard procedures for conducting bioassays with fathead minnows have been established by the United States Environmental Protection Agency (*6*), and procedures for acquisition and culture of research fish are also available (*13*). Fathead minnows adapt readily to fish feeds prepared commercially. Because of their small size, the feed for newly hatched fry must be extremely finely ground meal. In our tests, fish are fed live cultured brine shrimp until they are 15–20 days old at which time a commercial trout fry feed (Silver Cup Salmon) is pulverized and fed with the brine shrimp. When large enough (about 45 days), fish are fed dry feed twice each day with a supplement of live brine shrimp once each week.

PAST STUDIES

Although many reports are available that describe the acute, partially chronic, and chronic toxicity to fathead minnows of herbicides, pesticides, heavy metals, and various organic compounds, we found no published reports involving carcinogenicity testing to toxicants for this species. In vitro fish cell lines developed by Wolf and Quimby (*14*) have been used by some investigators to study the effect of carcinogens in vitro. Bourne and Jones (*15*) studied the effect of DMBA on RTG-2 cells and epithelioid cells of the fathead minnow. Mitotic indexes demonstrated that the RTG-2 fibroblastic cells were distinctly more sensitive to DMBA than were the epithelioid but did not show the multinucleation shown by the latter cells. These authors concluded that the action of DMBA on both cell lines confirmed that they are non-neoplastic.

Thornton et al. (*16*) studied the in vitro metabolism of benzo[*a*]pyrene by bluegill fry-2 and fathead minnow cell lines. Their results show that cells of both lines metabolize a high proportion of this polycyclic hydrocarbon to the 7,8,-diol-glucuronide and thus form a water-soluble conjugate of a proximate carcinogenic metabolite of benzo[*a*]pyrene, which could be transmitted to various tissues within the fish or throughout the aquatic environment.

ADVANTAGES AND SPECIAL PROBLEMS RELATED TO FATHEAD MINNOWS

The fathead minnow occupies a broad natural range from the prairie provinces of Canada and northern United States between the Rockies and Appalachians south to Tennessee, Arkansas, and northern Mexico (*17*). Stocks of fathead minnows are also available from commercial bait farms. Fish used in our studies were captured from a farm pond in eastern Montana.

Although fathead minnows can withstand considerable physical stress in the wild, they sometimes suffer diseases associated with the bacterium *Aeromonas liquefaciens,* the digenetic trematode *Gyrodactylus* sp., and the parasitic copepod *Lernea.* From diseased fish in the laboratory, we have identified the protozoans, *Costia* sp. and *Chilodenella* sp., more often the latter. We have also isolated *A. liquefaciens* and *Pseudomonas fluorescens* from diseased fish. Outbreaks of disease are usually associated with elevated temperatures or crowded conditions, or both. Brungs (*11*) found increased incidence of disease among fathead minnows reared at elevated temperatures.

In wild populations, most males and up to 90% of the females may die after spawning at 2 years; a few fish survive the third year (*4*). Under laboratory conditions, however, broodfish spawn during their first year of life and experience little postspawning mortality.

HYPERPLASTIC LESIONS OF THE PRIMITIVE MENINX INDUCED BY AMMONIA

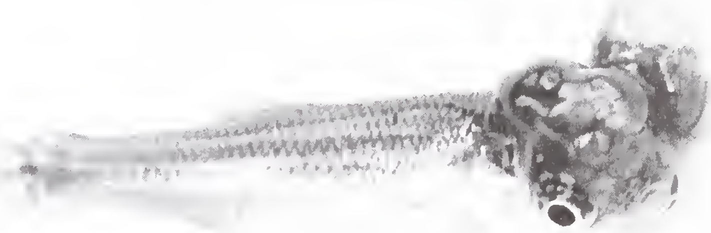
A test, conducted by investigators at the Fisheries Bioassay Laboratory, Montana State University, Bozeman, determined the effect on fathead minnows of chronic exposure to ammonia. Cultural procedures are similar to those described by the Environmental Protection Agency (*6*).

Fish were exposed for 11 months to 5 concentrations of ammonia within the range 0.639–2.17 mg NH_3/liter, i.e., 9.36–30.0 mg NH_3-N/liter, in 1 control and 5 test tanks (table 2). The temperature was approximately 25° C,

TABLE 2.—*Mean (and range) of water chemistry variables to which fathead minnows were exposed for 11 mo*

Tank No.	Temperature, degrees C	O_2, mg/liter	pH	Total NH_3-N, mg/liter	Un-ionized NH_3, mg/liter
1	25.6 (23.5–26.5)	6.7 (5.6–8.0)	8.08 (7.91–8.30)	0.07 (0.00–0.36)	0.006 (0.000–0.036)
2	25.1 (23.3–25.9)	6.6 (5.4–7.8)	8.02 (7.87–8.29)	9.36 (5.94–10.6)	0.639 (0.427–1.14)
3	25.0 (23.2–25.9)	6.6 (5.2–8.0)	7.98 (7.84–8.20)	12.2 (7.28–13.8)	0.815 (0.508–1.21)
4	25.2 (23.5–26.1)	6.6 (5.6–7.9)	7.97 (7.82–8.18)	16.4 (11.1–18.5)	1.07 (0.756–1.57)
5	25.0 (23.6–25.9)	6.7 (5.7–7.8)	7.96 (7.82–8.16)	19.7 (10.4–22.4)	1.25 (0.715–1.94)
6[a]	24.5 (23.1–25.7)	7.1 (6.2–7.9)	8.05 (7.84–8.16)	30.0 (27.7–32.6)	2.17 (1.36–2.99)

[a] Test in this tank was terminated after 133 days due to excessive mortality of fish.



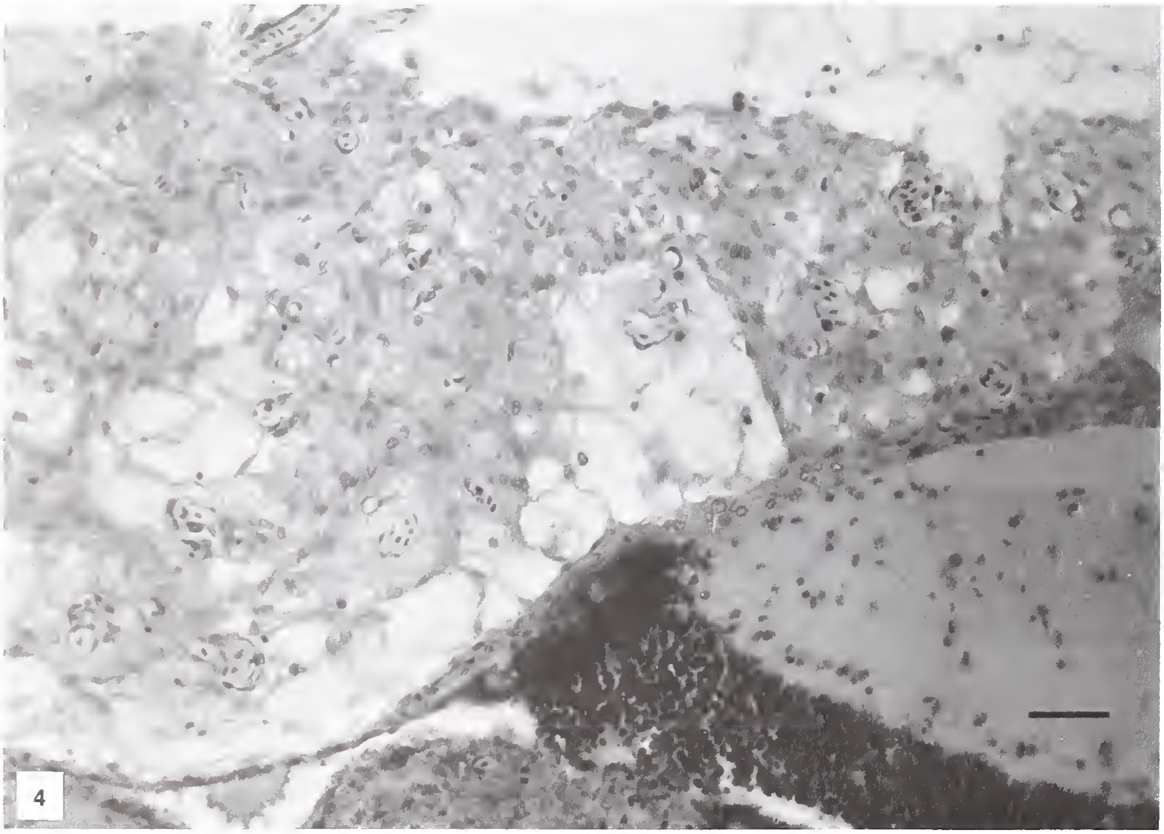


FIGURE 3.—Adult fathead minnow exposed to 1.25 mg NH_3/liter for 150 days showing massive growth on head. Eye has been misplaced ventrolaterally.

FIGURE 4.—Proliferation of cells from primitive meninx of fathead minnow exposed to 1.25 mg NH_3/liter for 60 days. H & E. Bar = 30 μm. × 400

oxygen concentration was 6.7 mg/liter, and the pH was 8.0 in all tanks. Ammonia in the form of ammonium chloride and dilution water was added to the tanks from a proportional diluter of the basic design of Mount and Brungs (*18*). The water exchange rate in each tank was approximately once every 2 hours. At the start of the test, fry between 1 and 5 days old were selected at random from a single pool and 50 were placed in each tank. At 60 days into the test, the number of fish in each tank was reduced to 15, except at the highest concentration on which fewer fish had survived. During the spawning period, the number of sexually mature males in each tank was reduced to no more than 4.

During the course of the study, growths were observed on the heads of several fish held at the 2 highest ammonia concentrations. Swollen darkened areas were noted on the heads of several fish held at lower concentrations. At 60 days when the fish were reduced in number, 7–15 fish were collected for histopathological examination from all but the highest concentration, and 2–8 fish were selected at the end of the study. Periodically, 8 additional fish exhibiting gross lesions were collected for histopathological examination. All fish were preserved in Bouin's fixative. Paraffin sections were cut at 5 μm and stained with H & E. Some sections were stained with Masson's trichrome, periodic acid-Schiff, and Alcian Blue. Frozen sections were cut from fresh and formalin-fixed material and stained with oil red O.

Grossly and histologically, the severity of lesions was positively correlated with ammonia concentration. One fish exposed to 1.25 mg NH_3/liter developed a lesion sufficiently massive to cause misalignment of the eyes (fig. 3). Histologically, the lesions appeared to be of a cell type that originated from the primitive meninx covering the brain (fig. 4). Hyperplastic tissue often completely surrounded the brain but was not observed around the spinal cord (figs. 5, 6). The cell type varied; many cells were swollen and contained small nuclei and a dense foamy cytoplasm. Rodlet cells were sometimes diffusely scattered throughout and often were concentrated at the periphery (fig. 7). Blood vessels were present throughout the hyperplastic tissue, and many had accumulations of melanin around their walls, thus giving a darkened appearance to the top of the head. Tissue was sometimes dense, especially in areas where it appeared to proliferate from the primitive meninx. Cells resembling adipocytes were scattered throughout. The hyperplastic tissue stained negatively for collagen, mucin, and mucopolysaccharides. An occasional cell stained positive for lipid, but most were negative. Macrophages containing degenerate, periodic acid-Schiff-positive debris were sometimes scattered throughout the tissue, and occasionally focal accumulations of macrophages were seen, especially in advanced lesions in which dystrophic calcification was also apparent.

Of the fish which exhibited gross lesions, 2 were transferred to a freshwater holding tank and maintained for approximately 1 month. During this time, the lesions noticeably regressed. Judged on the orderly appearance of tissues from lesions and evidence of their regression, the lesions observed are not considered to be neoplasms but connective tissue hyperplasia induced as a response to exposure to ammonia.

In summary, fathead minnows are easily maintained within the laboratory, can be induced to spawn their first year of life, have a short period of embryogenesis, and provide adequate numbers of eggs per spawning for testing. This species has proved to be extremely useful in toxicity testing of environmental contaminants and appears to have a potential usefulness in carcinogenicity testing.

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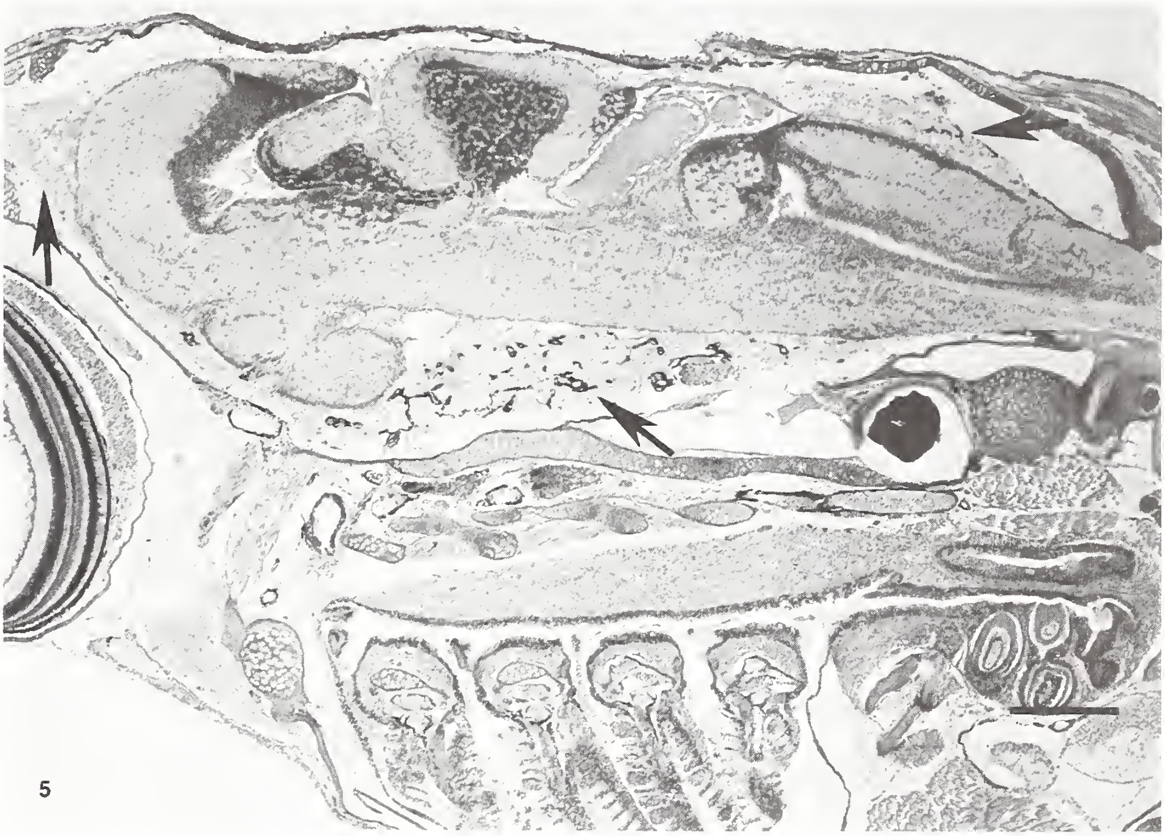

FIGURE 5.—Longitudinal section through brain of fathead minnow exposed to 1.25 mg NH_3/liter for 60 days. *Note* proliferation of tissue in different areas around the brain (*arrows*). H & E. Bar = 100 μm. × 60

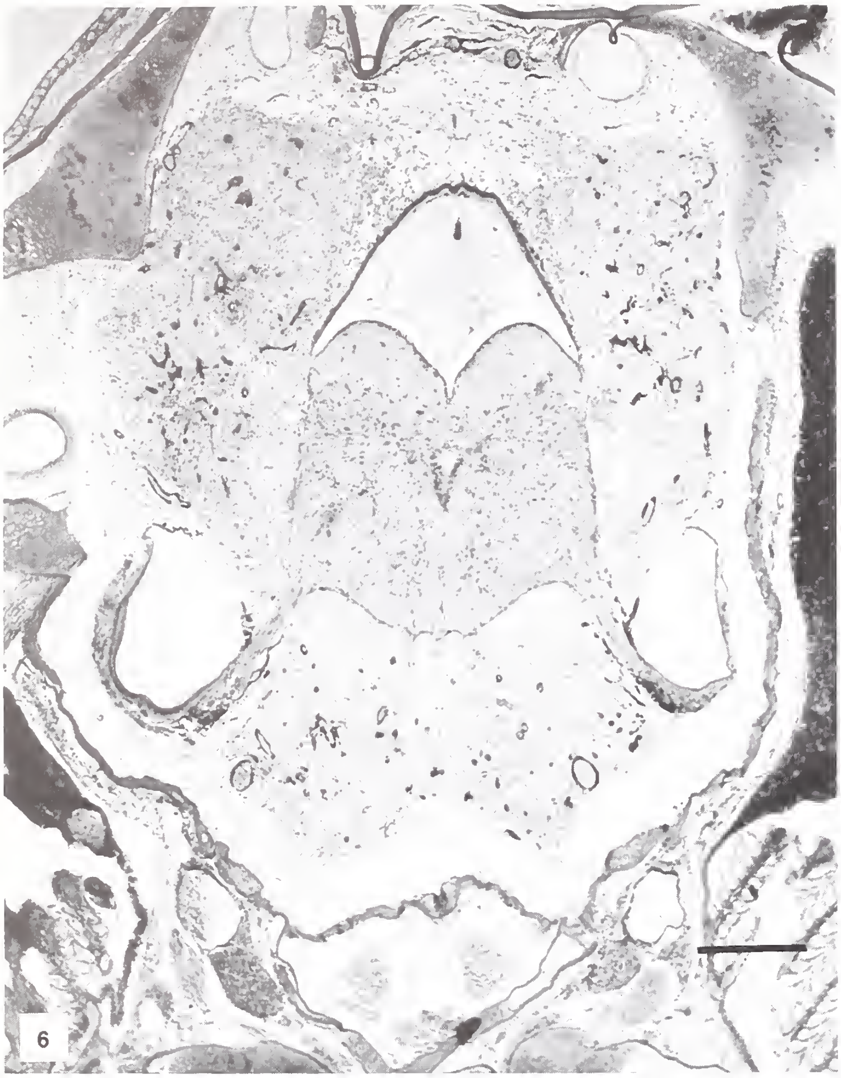

FIGURE 6.—Cross section through medulla of a fathead minnow exposed to 1.25 mg NH_3/liter for 60 days. *Note* extensive proliferation of tissue surrounding medulla and the accumulation of melanin around vessels scattered throughout proliferated tissue. H & E. Bar= 100 μm. × 60

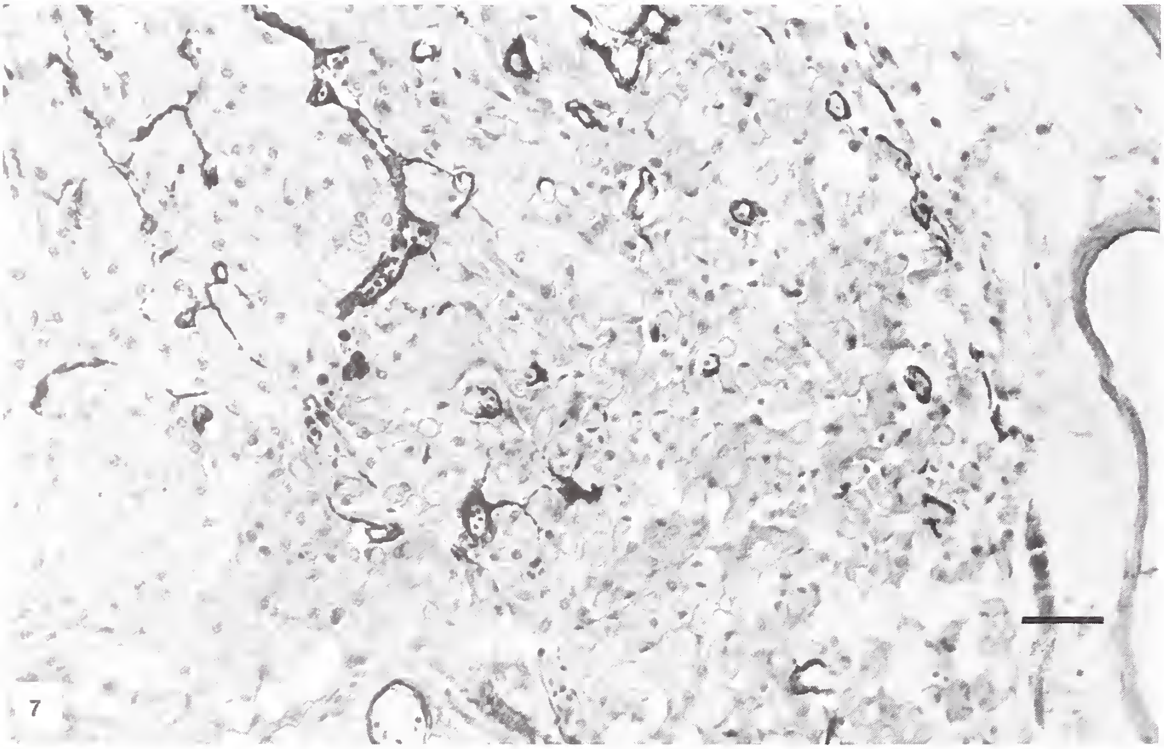

FIGURE 7.—Proliferated tissue containing swollen cells with small nuclei and foamy cytoplasm. Accumulations of rodlet cells are seen at the periphery of foamy cells in *right half* of photomicrograph. H & E. Bar= 30 μm. × 400

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Session II

Alternative Testing Systems With the Use of Fish

Chairman: **Benjamin Trump, M.D.**

Rainbow Trout Embryos: Advantages and Limitations for Carcinogenesis Research[1, 2, 3, 4]

Jerry D. Hendricks, Theodore R. Meyers, John L. Casteel, Joseph E. Nixon, Patricia M. Loveland, *and* George S. Bailey[5, 6]

ABSTRACT—Rainbow trout embryos are sensitive to the initiation of neoplasms in various tissues by brief exposures to solutions of water-soluble carcinogens. This characteristic was first demonstrated with the sparingly soluble liver carcinogen, aflatoxin B_1 (AFB_1). A 30-minute exposure of 21-day-old embryos (embryos hatch in 24–25 days at 12° C) to a 0.5-ppm aqueous solution of AFB_1 will result in approximately 65% of the survivors having at least 1 liver tumor, 1 year after treatment. The embryos are responsive to both AFB_1 dose and the length of exposure and become increasingly sensitive with increased embryonic age. We have used rainbow trout embryos to demonstrate the hepatocarcinogenicity of other aflatoxin metabolites and precursors; aflatoxicol, aflatoxin G_1, versicolorin A, and sterigmatocystin. In addition to mycotoxins, trout embryos are sensitive to several nitrosamine hepatocarcinogens including: dimethylnitrosamine, diethylnitrosamine, nitrosopyrrolidine, and 2,6-dimethylnitrosomorpholine. However, with the highly water-soluble nitrosamines, longer exposure times (up to 24 hr) are required. It is generally accepted that each of the above-named carcinogens requires metabolic activation to the ultimate carcinogenic form. This provides indirect evidence that the trout embryo is capable of cytochrome P-450-mediated metabolism. Finally, trout embryos are sensitive to the direct-acting carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. This compound produces tumors of the liver, stomach, kidney, and swim bladder, and a pronounced female-to-male sex reversal. Results to date have shown that the trout embryo is a sensitive, convenient, and economical whole animal model system with many distinct advantages for carcinogen testing and research. The major limitation has been the difficulty with exposure of the embryos to adequate doses of highly water-insoluble compounds. Alternate exposure techniques will be required for alleviation of this problem. — Natl Cancer Inst Monogr 65: 129–137, 1984.

The practice of exposing rainbow trout embryos to carcinogens was developed by investigators at Oregon State University to provide an alternative to dietary exposure for challenging experimental fish with known or suspect carcinogens. Initially, the many unique advantages of this technique were not foreseen, nor was the present popularity of using the embryos of trout as well as other fish species expected. The technique has been thoroughly described in several papers (*1–4*), but we restate the basic procedures to lend completeness to the subject of carcinogenesis research in fish in this monograph. Emphasis here will be placed on the advantages and limitations of the model system.

GENERAL DESCRIPTION OF PROCEDURES AND EQUIPMENT

Exposure of rainbow trout embryos to various carcinogens is routinely performed at the Food Toxicology and Nutrition Laboratory here at Oregon State University. Disease-free water is pumped from wells at a constant temperature of 12° C, a temperature that permits development of trout embryos from fertilization to hatching in 24–26 days. Sensitivity to carcinogens, which will be discussed later, is greatest near the end of embryonic development. To standardize our procedure and allow for comparison between treatments, we routinely use 21-day-old embryos for exposure to carcinogens. Embryos to be used for carcinogen exposure are selected from the stock supply (we make sure to select strong-eyed, vigorous embryos), counted (usually 200/treatment), and placed in our custom-built, embryo/sac-fry isolation unit (figs. 1, 2). This apparatus facilitates easy handling of the embryos or sac-fry in the inner egg cup and ensures that each group of fish has a separate water supply and drain to

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ABBREVIATIONS: AFB_1 = aflatoxin B_1; MNNG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DMNA = dimethylnitrosamine; DENA = diethylnitrosamine.

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[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Supported in part by Public Health Service grants ES00210, ES01926, ES00541, ES00550, and ES00092, from the National Institute of Environmental Health Sciences; grant CA20990 from the National Cancer Institute; and Cooperative Agreement grant CR809344-010 from the National Cancer Institute/Environmental Protection Agency Collaborative Program, Project No. 3, Effects of Carcinogens, Mutagens, and Teratogens in Non-human Species (Aquatic Animals), which is administered by the Gulf Breeze Environmental Research Laboratory, Gulf Breeze, Florida 32561.

[3] Animals were maintained under the guidelines set forth by the Oregon State University and in the "Guide for the Care and Use of Laboratory Animals" by the Institute of Laboratory Animal Resources, National Research Council.

[4] Technical Paper No. 6490, Oregon Agricultural Experiment Station, Oregon State University, Corvallis, Oregon 97331.

[5] Department of Food Science and Technology, Oregon State University. *Address reprint requests to* Dr. Jerry D. Hendricks.

[6] We thank Mrs. Duen Poocharoen and Mr. Theodore Will for their technical assistance.

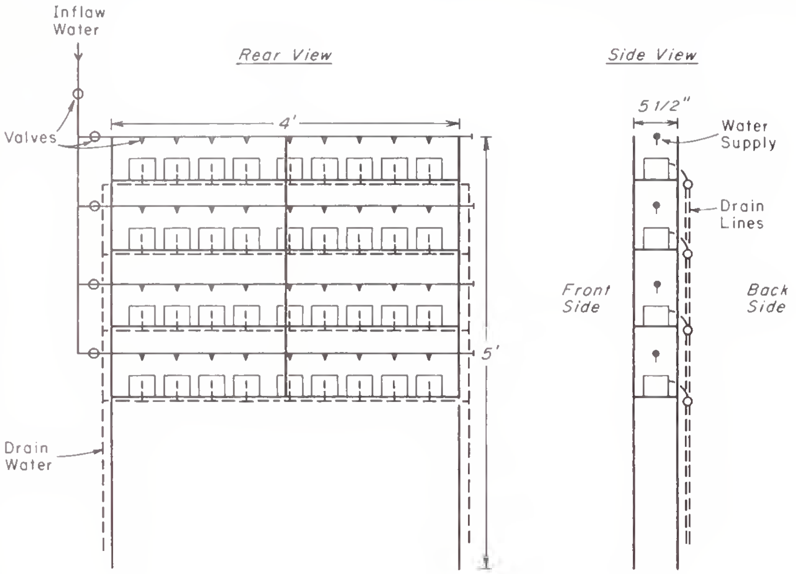

FIGURE 1.—Rear and side views of our embryo and sac-fry isolation unit which is used to house embryos and sac-fry before and after exposure to chemical carcinogens. Each unit (inner and outer cups) has its valved water source and drain.

eliminate any possible cross contamination with other groups.

The desired amount of carcinogen is added to a clean volumetric flask and made up to volume (usually 200 ml) with ambient temperature (12° C), well-aerated water. This alleviates the need for additional aeration during a 30- to 60-minute exposure. Longer exposures require the use of an air pump and air stones so that adequate aeration is ensured. The carcinogen solution is placed in a 500-ml glass container which is suspended in a running water bath to maintain the temperature. An inner egg cup containing 200 eggs is then removed from its individual water reservoir (outer cup) of the isolation unit, blotted on a paper towel to remove water, and set into the glass container with the carcinogen. Agitation of the egg cup on a regular schedule ensures continuous contact with the carcinogen. At the end of the desired time, the egg cup is removed from the solution and rinsed several times in containers of clean water and returned to its properly labeled outer cup in the isolation unit. The carcinogenic solution and rinse waters are combined; detoxification and disposition are done according to accepted procedures for the particular carcinogen.

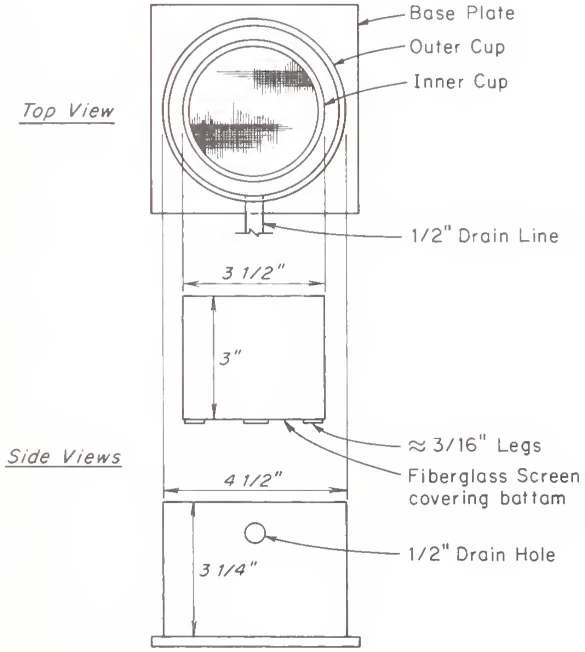

FIGURE 2.—Details of the inner and outer compartments of the individual holding facilities of the isolation unit. Both inner and outer cups are constructed of polyvinyl chloride pipe.

The remainder of the developmental period, hatching, and the sac-fry stage occur in the isolation unit. After hatching, each group of sac-fry is removed from its cup and placed in a shallow pan where egg shells, cripples, or unhatched eggs can be recorded and removed. As the time for swim-up and the onset of feeding approach, the sac-fry are placed in perforated plastic buckets suspended in larger tanks and fed small pieces of the Oregon Test Diet (*5*) or other diets depending on the postexposure protocol.

FACTORS AFFECTING EMBRYONIC RESPONSE TO CARCINOGENS

Age of Embryo

At 12° C, rainbow trout embryos reach the "eyed-egg" stage on day 14. At this time, embryonic development has progressed to the point that 1) the retinas are pigmented and externally visible, 2) epiboly is complete, which results in a layer of cells completely enclosing the yolk sac and making the embryo resistant to physical shock, and 3) the embryonic liver has budded from the gut as a discrete organ. Initial exposures of trout embryos to AFB_1 by Wales were performed on day 14, but we designed subsequent experiments to determine the age of greatest sensitivity. Using the egg cups described above, we could handle embryos during the tender stage, before day 14. Groups of 200 embryos were exposed on alternate days

TABLE 1.—*Hepatocellulur carcinoma incidence in rainbow trout treated on alternate days during embryonic development in 0.5 ppm AFB_1 for 1 hr*[a]

Age of embryo, days	Incidence[b]				Total No. of tumors, 12 mo	No. of tumors/liver at 12 mo		Average tumor diameter, mm, at 12 mo	
	10 mo		12 mo						
	No.	Percent	No.	Percent		Means ± SD	*n*	Means ± SD	*n*
1	0/25	0.0	0/59	0.0	0	0.0		0.0	
3	—	—	2/53	3.8	2	1.0 ± 0.0	2	3.0 ± 0.0	2
5	4/48	8.3	11/58	19.0	14	1.3 ± 0.5	11	5.3 ± 4.8	14
7	3/39	7.7	11/55	20.0	12	1.1 ± 0.3	11	2.9 ± 1.2	12
9	5/35	14.3	11/62	17.7	11	1.2 ± 0.6	11	3.4 ± 2.3	13
11	4/30	13.3	11/60	18.3	13	1.2 ± 0.4	11	4.2 ± 3.6	13
13	1/45	2.2	10/60	16.7	13	1.3 ± 0.5	10	3.2 ± 1.6	13
15	11/52	21.2	19/60	31.7	29	1.5 ± 0.8	19	4.1 ± 3.5	29
17	13/39	33.3	29/63	46.0	46	1.6 ± 1.4	29	4.2 ± 2.2	46
19	18/42	42.9	29/59	49.2	42	1.4 ± 0.9	29	6.2 ± 4.6	42
21	20/36	55.5	33/55	60.0	48	1.5 ± 0.9	33	4.2 ± 2.8	48
23	20/35	57.1	35/60	58.3	48	1.4 ± 0.6	35	6.6 ± 4.4	48
Control	0/40	0.0	0/60	0.0	0	0.0		0.0	

[a] Data are from Wales et al. (*1*).

[b] Values are No. of trout with tumors/total No. of trout. *Dash* indicates no sample was taken.

(1, 3, 5, . . . 23) throughout the developmental period to freshly prepared 0.5-ppm solutions of AFB_1 for 1 hour. Embryos were handled as described above and inspected for tumor development 10 and 12 months after exposure. Table 1 contains the results of this experiment and shows that sensitivity is greatest near the end of the developmental period (*1*). We also demonstrated (table 2) that hatched sac-fry were more sensitive to AFB_1 carcinogenesis than are embryos but that the lethality of AFB_1 was much greater too (*2*). This increasing sensitivity with age is probably due primarily to an increased functionality of the embryonic trout liver (table 3) which is required to metabolize AFB_1 to its active carcinogenic form (*2*). Even though sac-fry appear to be more sensitive to tumor formation than do embryos, the embryo stage still has many advantages, such as ease of handling and resistance to lethal effects, that favor its use over sac-fry in most instances.

Carcinogen Concentration

The dose responsiveness of rainbow trout embryos has been repeatedly demonstrated with several different carcinogens. Initially, Wales et al. (*1*) discovered that a concentration of 1 ppm AFB_1 for a 1-hour exposure was lethal to all the 14-day-old embryos tested. An hour treatment to 0.5 ppm AFB_1, however, resulted in low mortalities and a 42% incidence of hepatocellular carcinomas in 1 year. Later, we (*2*) conducted a dose–response experiment with AFB_1 that resulted in a definite dose-related response to liver tumors after 1 year (table 4). A similar response was observed after exposure of trout embryos to a range of concentrations of the direct-acting MNNG; only with this carcinogen, the dose–response was observed (*7*) with liver as well as kidney tumors (table 5). Because dose–response data are also available for versi-

TABLE 2.—*Increased sensitivity of rainbow trout to the lethal and carcinogenic effects of AFB_1 treatment*[a]

Age[b]	Treatment	Cumulative 90-day mortality	Liver tumor incidence[c]	
			12 mo	Percent
14[d]	AFB_1	27	57/119	48
	Sham	18	1/118	0.8
21[d]	AFB_1	39	79/120	66
	Sham	41	0/107	0
28[e]	AFB_1	265	56/102	55
	Sham	25	0/120	0
35[e]	AFB_1	144	38/58	66
42[f]	AFB_1	157	40/45	88
	Sham	3	0/120	0

[a] Data are from Hendricks et al. (*2*). Treatment consisted of 0.5 ppm AFB_1 for 1 hr as the age of the embryo sac-fry increased from 14 days postfertilization to yolk sac reabsorption.

[b] Age of embryos or sac-fry are in days postfertilization.

[c] Results are expressed as No. of trout with tumors/total No. of trout.

[d] Embryos were at eyed-egg stage.

[e] Hatching was complete, sac-fry stage.

[f] Yolk sac was absorbed, fry stage.

TABLE 3.—*Effect of age on liver mixed function oxidase activities in rainbow trout*

Mixed function oxidase parameter	Sac-fry[a]	Age of fish[b]	
		6 mo	18 mo
Cytochrome P-450[c]	0.08	0.38	0.44
Cytochrome *c* reductase[d]	26.10	38.05	38.73
Benzy[α]pyrene monooxygenase[d]		0.00	0.18
O-Demethylase[d]		0.42	3.01

[a] Data are from Hendricks et al. (*6*).

[b] Data are from Hendricks et al. (*2*).

[c] Values are expressed in nanomoles/milligram microsomal protein.

[d] Values are expressed in nanomoles/milligram microsomal protein per minute.

TABLE 4.—*Effect of* AFB_1 *concentration on the hepatocellular carcinoma incidence in rainbow trout treated for 1 hr as 21-day-old embryos*[a]

Concentration of AFB_1, ppm	Lot No.	Hepatocellular carcinoma incidence[b]					
		9 mo		Percent	12 mo		Percent
0.05	1	4/41	9/81	11	18/60	32/120	27
0.05	2	5/40			14/60		
0.1	1	6/39	16/76	21	31/60	58/118	49
0.1	2	10/37			27/58		
0.2	1	15/40	31/83	37	32/60	74/119	62
0.2	2	16/43			42/59		
0.5	1	11/29	21/62	34	42/60	79/120	66
0.5	2	10/33			37/60		
Control[c]	1	0/32	0/65	0	0/54	0/107	0
Control[c]	2	0/33			0/53		

[a] Data are from Hendricks et al. (*2*).

[b] Results are expressed as No. of trout with tumors/total No. of trout.

[c] Well water sham was used as the control.

colorin A (*8*), DMNA, and DENA (unpublished data), we are convinced that dose responsiveness is a predictable and reproducible characteristic of the embryo exposure model.

Length of Exposure

Wales et al. (*1*) discovered little or no difference between exposure of trout embryos to 0.5 ppm AFB_1 for 1 or 2 hours. Later, Hendricks and associates (*2*) showed that, with AFB_1, an optimal exposure time is apparent beyond which no increase and even a decrease in tumor incidence occurred (table 6). This may result from an interplay between the carcinogenic and lethal responses because a 30-minute exposure to this dosage results in essentially no mortalities, whereas a 1-hour exposure does cause some deaths. Perhaps some of the more sensitive fish that would develop tumors die and cannot be included in the final tumor incidence. Apparently, this variable would need to be established for each compound, inasmuch as the solubility of the compound, which will be discussed in the next section, greatly affects the time required for a response to occur.

Water Solubility of Carcinogen

The solubility characteristics of AFB_1, i.e., a lipophilic molecule that is only sparingly soluble in water, appear to be ideal for a rapid uptake from a water solution into the lipid-rich rainbow trout egg. Likewise, MNNG readily moves across the chorion barrier into the trout egg. However, highly water-soluble compounds, e.g., DMNA and DENA, move slowly into the high-lipid content trout egg. In fact, early exposure of these compounds to trout embryos, with the standard 1-hour treatment that was so effective with AFB_1, failed to produce any tumor response and made us (*2*) question their carcinogenicity to trout embryos (table 7). Doses of DMNA as high as 10,000 ppm were ineffective in producing tumors in trout exposed for 1 hour as 14-day-old embryos. Our decision to extend the exposure time to DMNA to 24 hours, using an air pump and air stone for aeration, resulted in a positive response, and subsequent experiments with DMNA and DENA repeatedly verified that the 24-hour exposure to water-soluble nitrosamines will result in a positive response. Thus the problems experienced with highly water-soluble compounds can be overcome by extension of the exposure time. The opposite problem, i.e., exposing trout embryos to highly water-insoluble carcinogens, is not so easily handled. Some compounds, such as benzo[*a*]pyrene are so water insoluble that an adequate dose cannot be achieved in a water solution.

CARCINOGENS TESTED TO DATE

The number of actual or suspect carcinogens tested to date in this system is small compared with the numerous compounds tested in rodent systems because of limited

TABLE 5.—*Effect of concentration on the carcinogenicity of MNNG to rainbow trout exposed for 1 hr as 21-day-old embryos*

MNNG concentration, ppm[a]	9-mo tumor incidence[b]				12-mo tumor incidence[b]			
	Liver	Percent	Kidney	Percent	Liver	Percent	Kidney	Percent
Control	0/40		0/40		0/60		0/60	
10	0/40		0/40		4/60	6.6	1/60	1.6
30	13/40	32.5	1/40	2.5	36/60	60	3/60	5.0
100	21/40	52.5	3/40	7.5	61/72	85	5/72	7.0

[a] Data are from Hendricks et al. (*7*).

[b] Results are expressed as No. of trout with tumors/total No. of trout.

TABLE 6.—*Effects of length of exposure time on carcinogenic response in trout exposed to 0.5 ppm AFB$_1$ as 21-day-old embryos*[a]

Exposure time, min	Hepatocellular carcinoma incidences[b]			
	9 mo	Percent	12 mo	Percent
15	14/79	19	58/119	49
30	37/85	44	86/118	73
60	21/62	34	79/120	66
Control	0/65	0	0/107	0

[a] Data are from Hendricks et al. (*2*).

[b] Results are expressed as No. of trout with tumors/total No. of trout.

funding, facilities, and staff here, where, until just recently, all this research has been done. The compounds tested fall into 2 main groups: 1) the aflatoxin precursors and metabolites, and 2) the nitrosamines. The aflatoxins tested so far include: 1) AFB$_1$, 2) aflatoxicol, 3) aflatoxin M$_1$, 4) aflatoxin Q$_1$, 5) aflatoxin B$_{2a}$, 6) aflatoxin G$_1$, 7) sterigmatocystin, and 8) versicolorin A, as shown in tables 8 and 9. Usually, the response of embryos to these compounds has been similar to that of fingerling trout to dietary exposure. The exceptions have been aflatoxin M$_1$, to which the embryo response was much lower than was the dietary response (*10*), and aflatoxin Q$_1$ with no response seen with the embryo exposure. For aflatoxin Q$_1$, the dose method for embryo exposure was probably inadequate to elicit a response, based on the dietary level that gave a positive response. Aflatoxicol appears to be more active when used in embryo exposure than in dietary testing, as shown in table 8 (*11*).

Nitrosamines or nitrosamides tested to date include DMNA, MNNG, dibutylnitrosamine, and ethylnitrosourea (tables 5, 7). Several others, including DENA, 2-6-dimethylnitrosomorpholine, nitrosopyrrolidine, and nitrosomorpholine have given positive responses for liver tumors, but the data have not been published.

Recent exposure of trout embryos to methylazoxymethanol acetate produced neoplasms of the liver, kidney, and stomach (*12*).

UNIQUE ADVANTAGES OF THE EMBRYO EXPOSURE MODEL

In discussing the advantages of a technique or model, we need a reference point against which comparisons are being made. Some of the advantages will be in reference to exposure of trout to carcinogens added to food, whereas others will use the common laboratory rat or mouse as a reference point. Advantages, in addition to those mentioned

TABLE 7.—*Carcinogenic response of rainbow trout to various nitrosamines and nitrosamides by exposure of 21-day-old embryos*[a]

Compound	Concentration of treating solution, ppm	Length of exposure, hr	Hepatocellular carcinoma incidence, 12 mo[b]	Percent
DMNA	500	24	10/60	17
"	1,000	1	0/60	0
"[c]	"	1	1/60	2
Dibutylnitrosamine	"	1	0/60	0
"[c]	"	1	0/60	0
Sham control	Well water	1	0/60	0
Ethynitrosourea	25	1	0/60	0
"	100	1	0/60	0

[a] Data are from Hendricks et al. (*2*).

[b] Results are expressed as No. of trout with tumors/total No. of trout.

[c] Exposure included a 15-min pretreatment in a 3% sodium chloride solution.

TABLE 8.—*Hepatocarcinogenicity of AFB$_1$ and its metabolites in rainbow trout exposed as embryos*[a]

Metabolite tested	Age of embryos days	Length of exposure, min	Concentration of exposure solution, ppm	Liver cancer incidence[b]			
				12 mo	Percent	15 mo	Percent
AFB$_1$	14	60	0.5	53/100	53	—	
Aflatoxicol	14	"	0.5	58/100	58	—	
Aflatoxin M$_1$	21	30	5.0	0/30	0	1/32	3
Aflatoxin Q$_1$[c]	14	60	1.0	0/200	0	—	
Aflatoxin B$_{2a}$	14	"	4.5	0/100	0	—	
Aflatoxin G$_1$	21	30	5.0	2/30	7	7/30	23

[a] Data are from Hendricks et al. (*2*).

[b] Results are expressed as No. of trout with tumors/total No. of trout. Dash indicates no tumors were observed.

[c] Data are from Hendricks et al. (*9*).

TABLE 9.—*Carcinogenicity of versicolorin A, sterigmatocystin, and AFB$_1$ to rainbow trout embryos*[a]

Compound	Dose, ppm[b]	Age of embryo, days	Liver cancer incidence, 12 mo[c]	Percent	Potency relative to AFB$_1$[d]
Sterigmatocystin	0.5	14	13/100	13	0.245
AFB$_1$	0.5	"	53/100	53	1.000
Sham control	—	"	0/100	0	0.000
Versicolorin A	5	21	14/33	42	0.063
Versicolorin A	25	"	40/59	68	0.020
AFB$_1$	0.5	"	41/60	68	1.000
Sham control (3% dimethyl sulfoxide)	—	"	0/60	0	0.000

[a] Data are from Hendricks et al. (*8*). Length of exposure was 1 hr.

[b] Dash indicates no mycotoxin treatment was given.

[c] Results expressed as No. of trout with tumors/total No. of trout.

[d] This potency was arbitrarily set at 1.000.

here, will probably become evident as more people use the model. The following are easily recognizable at the present time:

Passive Uniform Exposure

In a comparison of the 2 types of exposure, dietary and embryo, we believe that exposing the embryo permits a more uniform passive contact with the carcinogen; two sets of data from our laboratory lend support to this conclusion. The first data set includes the results of 21-day-old rainbow trout eggs exposed to a 0.5-ppm solution of [^{3}H]AFB$_1$, with a specific activity of 2.27 μCi/μg AFB$_1$, for 1 hour, rinsed and placed back in flowing well water. After 0.5, 2, 4, 8, and 24 hours, samples of 10 eggs were removed, weighed separately, and prepared for scintillation counting. Table 10 contains the average disintegrations/milligram of egg ± SE at the various periods. These values indicate a uniform uptake of AFB$_1$/egg, but at this time we lack quantitative values for the amount of AFB$_1$ incorporated into individual fish from dietary exposure for comparative purposes.

Secondly, indirect evidence from feeding trials and embryo exposures to AFB$_1$ indicate that a more uniform dosing occurs from embryo exposure. A social hierarchy is established in each tank of fish with more food being consumed by the dominant and less by the less dominant fish. This results in not only differential growth but also differential exposure if a carcinogen is being fed. Tumor incidence data illustrate that most of the tumor-bearing fish in a dietary study are the larger individuals, whereas few of the small fish have tumors. Thus tumor incidence is a direct reflection of the carcinogen dose consumed and is not necessarily related to individual sensitivity or resistance to a standard dose. However, a different result occurs in fish exposed to AFB$_1$ as embryos. A size distribution occurs similar to that in diet-exposed fish, but tumor incidence is much less dependent on fish size. In other words, a greater number of small fish and a smaller number of large fish have tumors, so that the tumor incidence is more evenly distributed throughout the size classes. We interpret these data to mean that a more uniform dose per individual is achieved by embryo exposure before the effects of diet consumption and resulting growth.

TABLE 10.—*Uptake of [^{3}H]AFB$_1$ by individual rainbow trout eggs*[a]

Time after treatment, hr	Average egg wt, g	Disintegrations/min/mg ± SE[b]	± SE
0.5	59.4±6.2	136±6.3	6.3
2	58.2±5.6	133±1.9	1.9
4	60.9±4.9	136±6.6	6.6
8	61.2±5.2	123±3.5	3.5
24	59.0±5.5	115±5.1	5.1

[a] Data are unpublished and from our laboratory.

[b] Values are average disintegrations/minute/milligram ± SE, $n = 10$.

Minimal Carcinogen Requirement

This exposure technique can probably achieve an adequate exposure for a larger number of animals to a given, small amount of carcinogen than any other method available. This has obvious advantages of monetary savings for expensive compounds, and it may result in the ability for one either to conduct a meaningful exposure or not because often only limited quantities of certain compounds are available and other types of exposures are not feasible.

Safety and Clean-up Convenience

The need for only minimal equipment and space makes containment and clean-up of contaminated objects an easy procedure. For a given exposure, only the volumetric flask, egg cup, solution container, and rinse dishes need to be contaminated. These are decontaminated according to accepted procedures with the waste solution and rinse water. If the compound is volatile, the entire procedure can be conducted in a fume hood for safety precautions. Dietary exposure requires more and larger equipment for preparation, storage, and feeding; consequently, clean-up and containment are more difficult (though not impossible). In addition, small amounts of carcinogen are unavoidably brought into contact with the water during feeding, which results in greater escape into the drainage system.

Adequacy of Single Exposure

Most of the compounds which are carcinogenic by prolonged dietary exposure are also carcinogenic by single embryo exposure, and, therefore, repeated exposure is not usually necessary in the trout model. The advantages of single versus multiple exposures are obvious: This feature is particularly valuable to the researcher from both safety and convenience standpoints. Dietary exposure requires handling the toxic diet on a daily basis, increasing the odds for personal exposure and contamination. However, some compounds, such as benzo[*a*]pyrene, do appear to require prolonged dietary exposure to elicit response in the trout model and are unsuitable for embryo testing.

Uniqueness for Embryonal Carcinogenesis

Embryonal carcinogenesis can be studied without several interfering factors present in mammalian systems. Because trout embryos are extrautero, they can be manipulated various ways with respect to temperature, oxygen, other water quality variables, and various chemical treatments without the placental barrier. Fish embryos should provide a valuable alternative model for this rapidly growing area of research.

Desirability for Tumor Promotion and Inhibition Studies

The characteristic of liver tumor initiation from a single exposure, plus the low to nonexistent background tumor incidence in liver and other organs in rainbow trout, make trout embryos a desirable system in which one can study the effects and mechanisms of whole organ tumor promotors or inhibitors. Both preinitiation and postinitiation effects can be investigated without the complicating maternal influence found in mammals. Examples of postinitiation promotional effects by cyclopropenoid fatty acids and high dietary protein are included in tables 11 and 12, respectively (*3, 13*). An example of a preinitiation promotional effect of Aroclor 1254 on AFB$_1$ tumor initiation is shown in table 13 (*6*), in which preexposure to Aroclor was achieved by its inclusion in the maternal diet during oogenesis.

TABLE 12.—*Postinitiation promotional effects of high dietary protein on liver tumor incidence initiated in rainbow trout embryos*[a]

Protein level of diet, %[b]	Hepatic tumor incidence[c]			
	9 mo	Percent	12 mo	Percent
40	8/55	12	50/115	43
50	18/73	25	55/115	48
60	28/70	40	93/117	79
70	49/72	68	99/115	86

[a] Data are from Hendricks et al. (*13*). Exposure was for 30 min to 0.5 ppm AFB$_1$.

[b] Protein level was calculated from fish protein concentrate/gelatin mixture.

[c] Incidence figures are No. of tumor-bearing trout/total No. of trout sampled.

Unique Temperature Effects Model

One can conveniently manipulate environmental temperature before, during, or after carcinogen exposure in fish to determine the effects of temperature on cancer initiation and development. Egami et al. (*14*) demonstrated a distinct temperature effect (higher temperatures resulted in higher tumor incidence) on the development of liver cancer in the medaka (*Oryzias latipes*) when exposed as adults to DENA in the water. We conducted some preliminary experiments on the effects of temperature on embryo exposure to AFB$_1$ by exposing 3 groups of embryos to 0.5 ppm AFB$_1$ for 15 minutes at 12°, 15°, and

TABLE 11.—*Promotional effects of dietary cyclopropenoid fatty acids on the carcinogenicity of AFB$_1$ in rainbow trout exposed as 21-day-old embryos*

Treatment		Diet fed to fry	Hepatocellular carcinoma incidence[a]			
AFB$_1$ concentration, ppm	Length of exposure, min		9 mo	Percent	12 mo	Percent
0.5	15[b]	CD	14/79	19	58/119	49
"	"[c]	CD[d]	58/83	70	98/116	84
"	30[b]	CD	37/85	44	86/118	73
"	"[c]	CD[d]	74/81	91	97/111	87
"	60[b]	CD	21/62	34	79/120	66
"	"[c]	CD[d]	64/80	80	103/116	89
0.2	"[b]	CD	31/83	37	74/119	62
"	"[c]	CD[d]	69/83	83	104/118	90
0.1	"[b]	CD	16/76	21	58/118	49
"	"[c]	CD[d]	61/82	74	91/112	81
0.05	"[b]	CD	9/81	11	32/120	27
"	"[c]	CD[d]	30/81	37	103/116	89
Sham	"[b]	CD	0/65	0	0/107	0
Sham	"[c]	CD[d]	5/83	6	11/119	9

[a] *See* footnote *b*, table 4.

[b] Data are from Hendricks et al. (*2*).

[c] Data are from Hendricks (*3*).

[d] CD = control diet; we added 50 ppm cyclopropenoid fatty acids to the diet of fish given this concentration of AFB$_1$ at exposure time indicated.

TABLE 13.—*Hepatocellular carcinoma incidence in rainbow trout exposed to 0.5 ppm AFB$_1$*[a]

Treated trout	Hepatocellular carcinoma incidence[b]			
	9 mo	Percent	12 mo	Percent
Control female				
AFB$_1$, 30 min	15/40	38	39/59	66
Sham	0/40	0	0/58	0
Polychlorinated biphenyl-fed female				
AFB$_1$, 30 min	24/40	60	53/57	93
Sham	0/40	0	0/56	0

[a] Data are from Hendricks et al. (*6*). Female fed polychlorinated biphenyl was given a control diet ad libitum containing 200 ppm Aroclor 1254 for 2 mo before spawning.

[b] Results are expressed as No. of trout with tumors/total No. of trout.

18° C and then returning them to ambient 12° C water. Even this brief change in temperature during exposure produced a trend of increasing tumor incidence with increased environmental temperature (table 14). The potential for mechanism studies based on temperature shifts in this system is obvious.

Combined Carcinogenesis and Teratogenesis Model

Considerable evidence exists that exposure to mutagens and carcinogens before the completion of embryonic organogenesis results predominantly in teratological effects, whereas exposure after the completion of organogenesis produces largely neoplasia (*15*). Thus depending on the timing of exposure relative to embryonic development, either or both effects may be produced in the offspring. The trout embryo model should provide an easily manipulated system for teratogenesis studies.

Economical, Rapid Test System

Trout embryos provide an economical system in which one can test the potential carcinogenic effects of new compounds. Tumor studies in rainbow trout embryos can be conducted at about one-twentieth of the per animal husbandry cost of rodents, a factor that makes trout a highly desirable test animal. In addition, trout are large enough to provide ample tissue for metabolic studies on the mechanisms of action of carcinogens.

TABLE 14.—*Effects of temperature on the initiation of hepatic tumors in rainbow trout embryos by a 0.5-ppm solution of AFB$_1$*[a]

Exposure temperature, degrees C, 15 min	Incidence of hepatocellular carcinomas[b]			
	9 mo	Percent	12 mo	Percent
12	14/40	35	37/60	62
15	17/40	42.5	41/60	68
18	25/40	62.5	41/60	68

[a] Data are unpublished results from our laboratory.

[b] Results are expressed as No. of trout with tumors/total No. of trout.

LIMITATIONS OF THE EMBRYO EXPOSURE MODEL

Exposure to Water-Insoluble Carcinogens

The major limitation that we have encountered with the trout embryo model is the inability to expose embryos to adequate doses of highly water-insoluble carcinogens. Thus by our standard methods, we have not achieved an adequate exposure to compounds, such as benzo[*a*]pyrene, 7,12-dimethylbenz(*a*)anthracene, cyclopropenoid fatty acids, etc. Perhaps the best way we can circumvent this limitation is to develop embryo or sac-fry injection techniques (Sonstegard RA: Personal communication) to achieve adequate exposure to these compounds.

Limited Availability of Eggs

We use a single strain (Shasta) of rainbow trout which spawn naturally only once a year. Without artificial manipulation, all our embryo exposures take place during December, the month of natural spawning. However, the use of salmon pituitary extract can induce accelerated gonad maturation and make gametes available at any time of the year, should this prove desirable for certain experiments.

Long Generation Time

A related limitation is the long generation time (2–3 yr or 1–2 yr with gonadotropin) compared with rodents or aquarium fish, which limits the frequency of performing crosses and backcrosses for examination of the genetics of the carcinogenic response in this model.

Facilities

Rainbow trout require more exacting rearing conditions than most other fish species. The need for well-aerated, cool (<16° C), and uncontaminated water prevents their efficient rearing in many locations. Again, this limitation can be overcome by the use of activated charcoal filtration, refrigeration units, and aeration devices, if necessary, but rearing costs and the economic advantages of trout may be lost in the process.

CONCLUSIONS

All animal models have their inherent advantages and disadvantages depending on the nature of the problem studied. We found the rainbow trout embryo model to be economical, convenient, and sensitive for both carcinogen testing protocols and other aspects of carcinogenesis research, such as inhibition and promotion studies. A number of other applications, some of which have been mentioned in this paper, should extend the usefulness of this model. The general concept of fish embryo exposure to carcinogens can be used with many different species, some of which may have unique characteristics that favor their use rather than trout for specific purposes. The major advantages of the trout system that may indicate its choice over other fish species for some studies are: its growth to large size (to provide ample tissue for extensive biochemical studies), its well-established husbandry and worldwide availability, and, perhaps most importantly, the wealth of background information accumulated over the past 17 years of development of the trout model for studies on mechanisms of chemical carcinogenesis and its modulation by genetic and dietary factors.

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Correlation of Epizootiological Observations With Experimental Data: Chemical Induction of Chromatophoromas in the Croaker, *Nibea mitsukurii*[1, 2]

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ABSTRACT—Chromatophoromas in the croaker nibe, *Nibea mitsukurii*, are common neoplasms in feral fish which inhabit the shallow water in a unique geographic distribution along the Pacific coast of Japan. We undertook surveys of the epizootiology of tumor-bearing fish at 25 sites. The highest tumor incidence occurred at the station near the mouth of the Kumano river and was 47% (1,415 of 2,991). The incidence at 2 adjacent survey stations located approximately 18 and 30 km away were 2.7 and 2.5%, respectively. At the other survey stations, no tumor incidences were recorded or they were less than 5%. During the course of experimental studies on the chromatophoromas using tank-reared nibe, we found that nifurpirinol (NP), a drug used for the treatment of fish diseases, might also induce the chromatophoromas in the fish, as well as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and 7,12-dimethylbenz[*a*]anthracene. Therefore, tank-reared nibe 5 months of age were divided into 4 groups of about 50 each and exposed to water containing 0, 0.5, 1, or 2 ppm NP, respectively, fourteen times for 1 hour each time. Three hundred nibe were kept as untreated controls. The incidences of chromatophore hyperplasia or neoplasia per group at 13 months were as follows: the untreated: 2.9% (6 of 204), 0 ppm: 5.3% (2 of 38), 0.5 ppm: 73% (36 of 49), 1 ppm: 87% (20 of 23), and 2 ppm: 100% (2 of 2). These and other results led us to believe that 1) NP is a carginogen, 2) nibe have a high susceptibility to induction of chromatophoromas by chemical carcinogens, and 3) some environmental chemicals are causal factors in the hyperendemic occurrences of the tumors in wild nibe. — Natl Cancer Inst Monogr 65: 139–154, 1984.

Studies of tumors in feral fish may have unique significance for cancer research in the following ways: 1) introductions to the discoveries of new problems and of new experimental systems; 2) animal models for use in correlative studies of epidemiology with experimental oncology; and 3) early detection of and monitoring systems for environmental carcinogenesis. With these 3 points as a basis, more than 25 species-specific neoplasms in feral fish have been studied epizootiologically (*1*). However, only a few induction studies of such kinds of tumors have been done with the exception of those on lymphosarcoma in pike (*2, 3*).

The chromatophoromas in the croaker nibe (*Nibea mitsukurii*), and koichi (*N. albiflora*) were first observed in 1973 (*4*).[11] In 1974, a group was formed in our country to investigate tumors in the feral fish which inhabit the Japanese coastal waters. Their main aims were to survey the geographic distribution and to determine the causal factors of the tumors (if possible) and whether the occurrence of some tumors in feral fish could be related to chemical pollutants in the water. Since then, problems concerning the croaker chromatophoromas were among the main subjects of collaborative studies (1974–76 and 1977–79) because the tumors are common neoplasms in wild fish in Japan. During the course of the group studies and thereafter, we and others performed the investigations listed in table 1.

In this paper, we are primarily concerned with epizootiological observations and chemical induction of chromatophoromas in the croaker nibe, *N. mitsukurii*.

ABBREVIATIONS: DMBA = 7,12-dimethylbenz[*a*]anthracene; MNNG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; NP = nifurpirinol or 6-hydroxymethyl-2-[2-(5-nitro-2-furyl)-vinyl]-pyridine; H & E = hematoxylin and eosin.

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[11] In the report (*4*), the first author misidentified the species of the fish. The species was reported as *Argyrosomus argentatus;* actually, it was *N. mitsukurii* or *N. albiflora.*

TABLE 1.—*Group studies, 1974–76 and 1977–79*

Studies	Investigators
Morphology of tumors in nibe and other species	Kimura I, Ito M, Nagayo T, et al.
Epizootiology of the tumors in nibe and other species	Kimura I, Taniguchi N, Kubota S, et al.
Ecology and population genetics of nibe	Taniguchi N, Okada T, Miyazaki Y, et al.
Chemical analysis and mutagenicity testing of environmental substances	Tomita I, Kinae N, Saito S, et al.
Search for infectious agents and in vitro studies of the chromatophoromas	Ishikawa T, Kimura I, Yoshizaki K, et al.
Establishment of hatchery-rearing techniques and developmental biology of nibe	Taniguchi N, Kumai H, Nakamura G, et al.
Chemical induction of the chromatophoromas in nibe	Kimura I, Kumai H, Taniguchi N, et al.
Comparison of incidences of chromatophoromas in hatchery-reared nibe derived from parental fish caught within high- and low-incidence areas, respectively	Kumai H, Kimura I, Nakamura G, et al.
Carcinogenicity testing in mice of mutagenic environmental substances and known chemicals that induced the chromatophoromas in nibe	Kimura I, Miyake T, Kitaori H, et al.

EPIZOOTIOLOGICAL OBSERVATIONS

Materials and Methods

Nibe (N. mitsukurii) and survey stations.—Nibe (*N. mitsukurii*) is a teleost which belongs taxonomically to the order of Percida and the suborder of Percina and the family Sciaenidae. It is a common fish inhabiting shallow waters along the Pacific coast from the middle to southwestern portions of Japan (from Tohoku district in Honshu to Kagoshima Prefecture in Kyushu) where the Black Current flows far offshore, affecting the smaller coastal currents. Our surveys were done at approximately 70 stations in Honshu, Shikoku, and Kyushu, and nibe were found at 25 stations which lay mostly close to estuaries of rivers along the Pacific coast. We could not catch any nibe on the Japan Sea side of Honshu, although several books on taxonomy state that the fish is also found there. In the Seto Inland Sea, koichi (*N. albiflora*) was encountered instead of nibe. The survey stations for the chromatophoromas in nibe and koichi are shown in figure 1.

Nibe inhabited mostly shallow waters less than 15 m deep over sand or gravel bottoms on the coast near the estuaries of rivers facing the open sea. They were rarely observed in waters with rock or mud bottoms. Other species of croakers dwelt on the mud bottoms of inner bays and those of the deep offshore areas. The spawning season of nibe is from April to June. One of us (N. T.) discovered that nibe inhabiting the area near stations I and R reached a mean body length of about 18, 25, 30, 34, and 37 cm at 1, 2, 3, 4, and 5 years after hatching, respectively, although wide ranges of variation were observed (*5*). Koichi spawn their eggs from May to August and grow faster than nibe (*6*). Little is known about the habitat of koichi in the Seto Inland sea. Several sport fishermen told us that they usually caught the fish at points near the tide way.

Samplings and observations.—Epizootiology of the 2 species of croaker, nibe and koichi, was surveyed 187 and 9 times at 25 and at 7 stations, respectively. Fishing methods varied. Groups of sport fishermen usually cooperated with us and showed or gave us all the fish they had caught by rod and line from the shore. We occasionally used professional fishermen with gill nets and also observed nibe which were caught by trawl nets or by those nets that had been set. The total body length of the fish was measured to the nearest millimeter. The location, size, and colors of the tumorous lesions were always recorded on a standardized data sheet. Whole-body pathological observations of nibe were made on 179 fish caught on 4 occasions for surveys in 1973 and 1976. At other times, only representative lesions or suspicious lesions were observed histologically or electron microscopically. We frequently observed scale rings to determine the age of the fish.

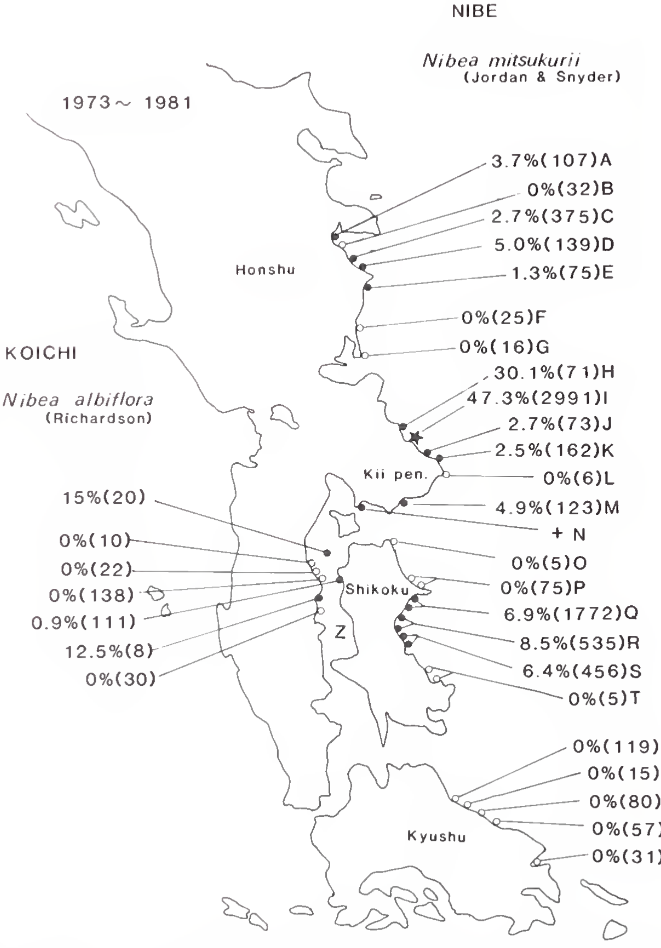


FIGURE 1.—Geographic distribution of chromatophoromas in the croakers: nibe and koichi, 1973–81. *Numbers in parentheses* are No. of effective fish observed.

Results

We observed the following 5 species of croakers: nibe (*N. mitsukurii*), koichi (*N. albiflora*), ishimochi or shiroguchi (*A. argentatus*), oonibe (*A. japonicus*), and kuroguchi (*A. nibe*). However, the chromatophoromas were seen only in 2 species of croaker, the nibe and koichi.

Morphological Characteristics of the Chromatophoromas in Nibe and Koichi

The chromatophoromas in both species were essentially similar and were macroscopically identified as black, gray, white, or mixed-color lesions on the skin (figs. 2, 3); they had a tendency to occur polycentrically on all portions of the integument. The smallest tumorous lesions resembled pigmented nevi of scale size (~4 mm in diameter) or fin-ray size (~5×2 mm). The large tumors, often more than 4 cm in diameter and over 10 mm thick, invaded adjacent muscle layers. Metastases into gills and other organs were observed occasionally. The black lesions were melanophoromas. The tumors were composed of spindle-shaped pigmented cells with long thin cytoplasmic projections. In the small lesions, the tumorous pigmented cells were almost like normal dermatochromatophores, grew parallel to the basal layer of the epidermis, and spread into the subcutaneous tissues. Occasionally, the neoplastic cells surrounded scales or infiltrated into the inner spaces between fin-ray bridge bones. The medium- and large-sized tumor cells had basophilic cytoplasm, were arranged in interlacing fascicles, and showed characteristic whorl, whirligig, or starburst patterns supported by a meshwork of collagen and reticulin fibers (figs. 4–6). Electron microscopically, the melanosomes were approximately 0.3 to 0.5 μm in diameter and varied in the amount from one cell to another (fig. 7). However, the larger tumors generally had less pigment. A characteristic feature of the tumor cells was that they had long thin cytoplasmic projections which retained dendritic features. The cytoplasmic projections of adjacent cells interlaced. For the most part, the nuclei were oval in small tumors but had irregular outlines in the cells of the larger ones. The cytoplasm contained a few oval-shaped mitochondria of approximately 0.3 to 0.5 μm in diameter, well-

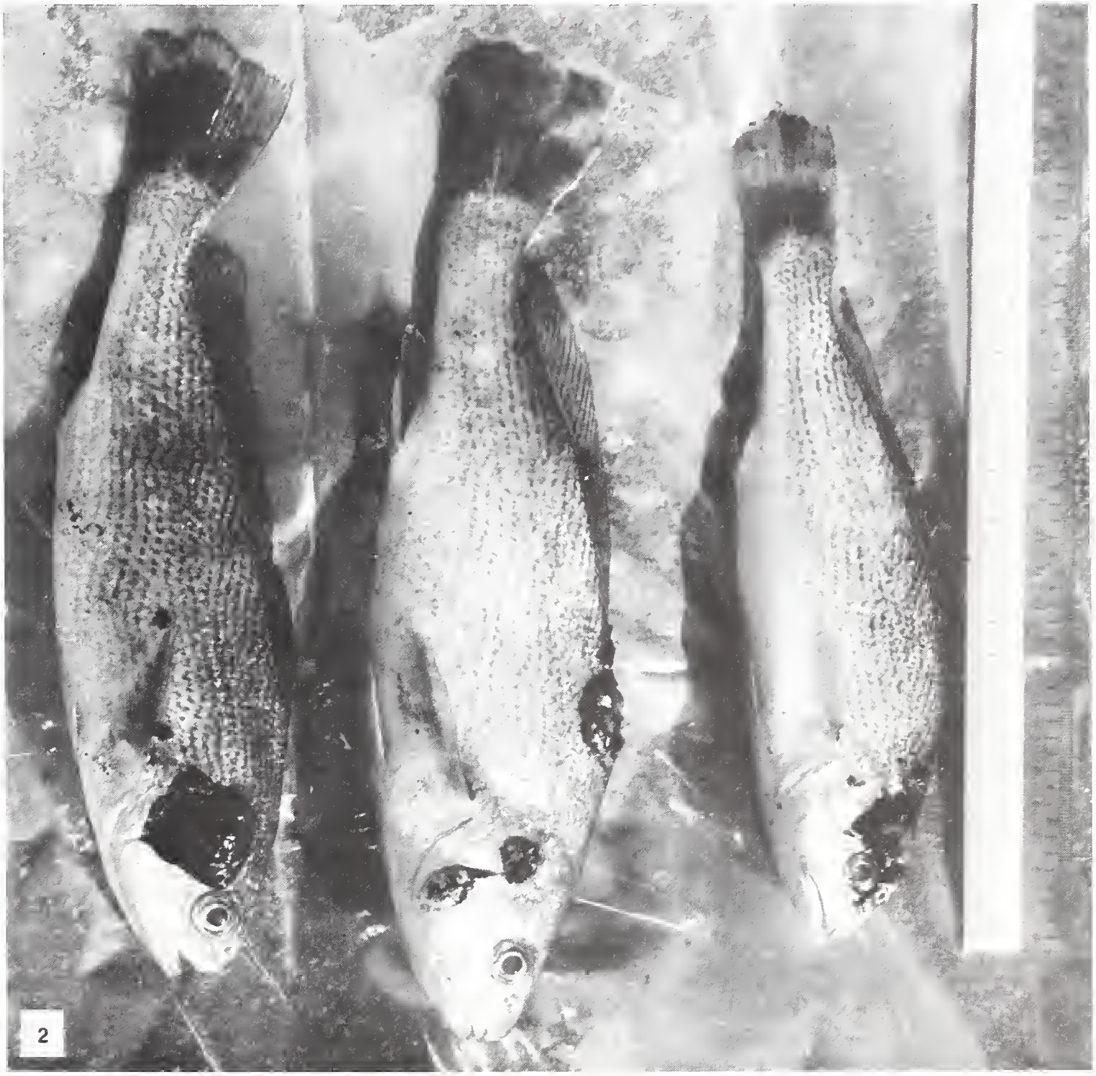


FIGURE 2.—Nibe, *N. mitsukurii*, caught at station I. *Note* multiple black lesions on skin of fish.

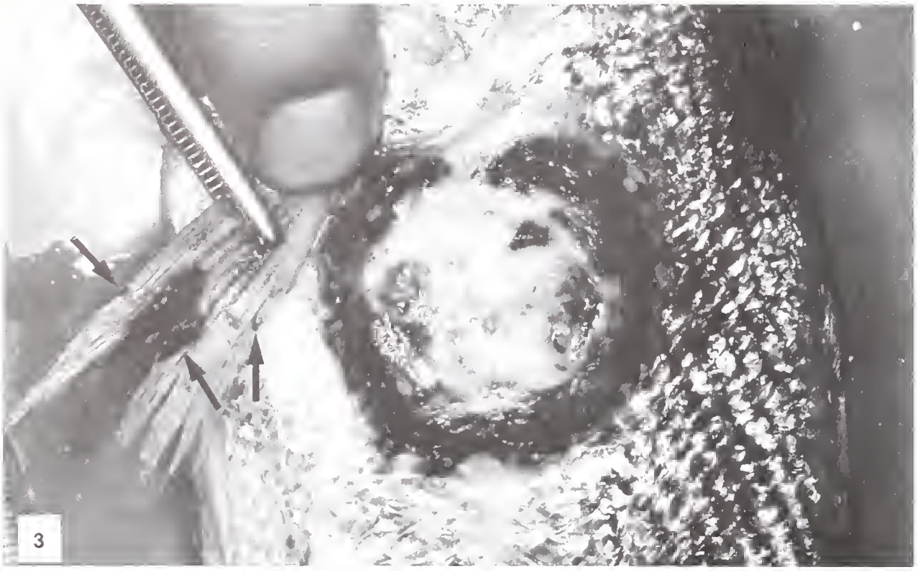


FIGURE 3.—Trunk and a pelvic fin of nibe. A large tumor on the trunk was a mixed lesion of melanophoroma, iridiphoroma, and poorly differentiated chromatophoroma. Small pigmented lesions (*arrows*) were observed on the fin in addition to large melanotic lesions.

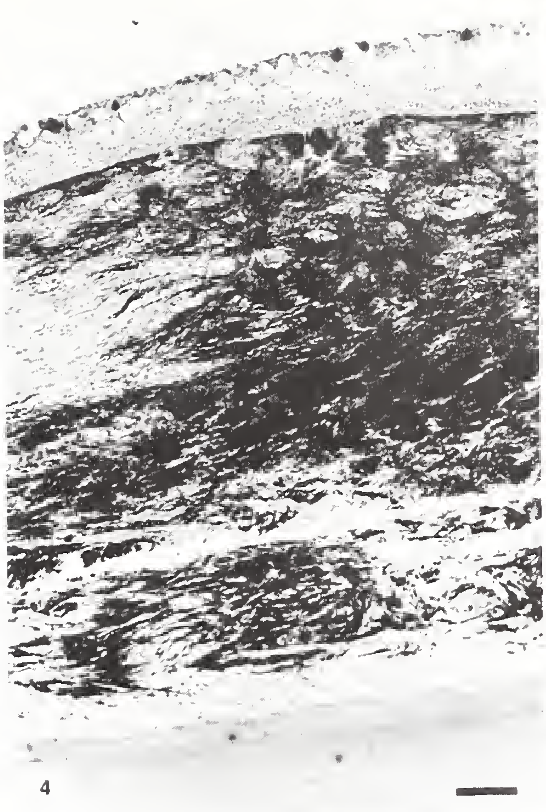
developed smooth endoplasmic reticulum, and pinocytic vesicles which were distributed especially around the nucleus and peripheral boundaries of the cell. Microtubules of 230 to 250 angstroms in diameter and bundles of fibers of approximately 100 angstroms were occasionally seen near the nucleus. However, rough endoplasmic reticulum and Golgi apparatus were not prominent in the neoplastic cells.

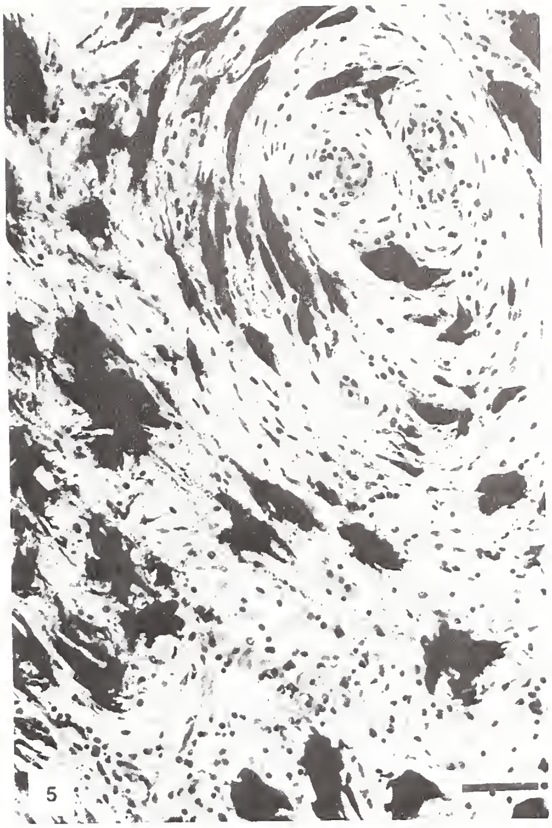
The white lesions were composed of iridophores which were characterized by brilliant cytoplasmic crystals (fig. 8). These crystals were birefringent and readily soluble in acids or alkalis corresponding to the chemical nature of guanine (*7*). Figure 9 shows an electron micrograph of the iridophoroma that was taken of a thin section without staining. The iridophoroma cells were characterized by numerous electron-dense platelets or laminated structures in their cytoplasm. The platelets or the laminated structures were limited by a single membrane and measured approximately 0.1 μm wide and 0.5 to 1.5 μm long. The electron-dense inner material of these structures was readily soluble in the solutions of uranyl acetate and lead nitrate, and the structure was identified as reflecting platelets (*8*). The ultrastructural characteristics of the melanophoroma and the iridophoroma cells were generally similar with the exception of the presence of melanosomes or reflecting platelets. Histological patterns of the iridophoromas were also similar to those of the melanophoromas, but the iridophoromas had fewer collagen fibers in their nests. Gray lesions were mostly mixed tumors of the melanophoroma and of the iridophoroma. In the portion of large tumors which invaded muscle layers, the tumor cells had thin fibrous cytoplasm and lacked pigment, although observations by electron microscopy have not been made on these portions.

The details of the morphological aspects of the pigmented lesions will be reported elsewhere. We assume that 1) the pigmented lesions occur first as a hyperplastic proliferation of dermatochromatophores which then develop into typical chromatophoromas containing cells arranged in interlacing fascicular, whorled, whirligig, or "star-burst" patterns, and 2) the various histological appearances correspond to the respective stages in tumor progression as well as to different kinds of chromatophores.

Geographic Distribution of Chromatophoromas in Nibe

The incidences of chromatophoromas in the nibe and koichi caught at the respective stations are shown in figure 1. Because no tumors were observed in the fish less than 209 mm in total body length, the fish larger than 210 mm were tentatively designated as effective fish, and the incidence of chromatophoromas was compared on the chart. The tumor incidences were less than 5% at the water areas from A to D and from E to G, which were located along the shore of Suruga Bay and facing the open sea, respectively. The fish caught at stations P, Q, R, and S along the coast of Tosa Bay in Shikoku showed relatively high tumor incidences (from 6.4 to 8.5%). No tumorous nibe were observed at stations U to Y located along the eastern coast of Kyushu. The geographic distribution of the chromatophoromas were unique to Kii Peninsula; about 47% (1,415 to 2,991) of effective nibe at station I developed chromatophoromas on their skin. The incidence at station H was also high, whereas it was significantly lower in the fish at stations J (2.7%) and K (2.5%), which were located 18 and 30 km away from station I, respectively. Figure 10 is the map of the coast between stations H and K and shows results of our survey concerning the habitat of nibe (Kimura I, Sakamoto T, Yoshizaki K: Unpublished data). The places where nibe were commonly caught were limited to the waters near estuaries of the rivers on sandy bottom less than 15 m depth. Another species of croaker, *A. argentatus,* was caught instead of nibe at the areas on the muddy bottom, such as the places 20 to 30 m deep at stations I and K, or in the inner bay near J. No croakers were caught at the areas with a rocky bottom. Thus the habitat of nibe was limited topographically and ecologically. Station H is located at another side of a continuous gravel shore of about 24 km from the estuary of the river at station I. There is no topographical barrier, such as a rock bottom, between





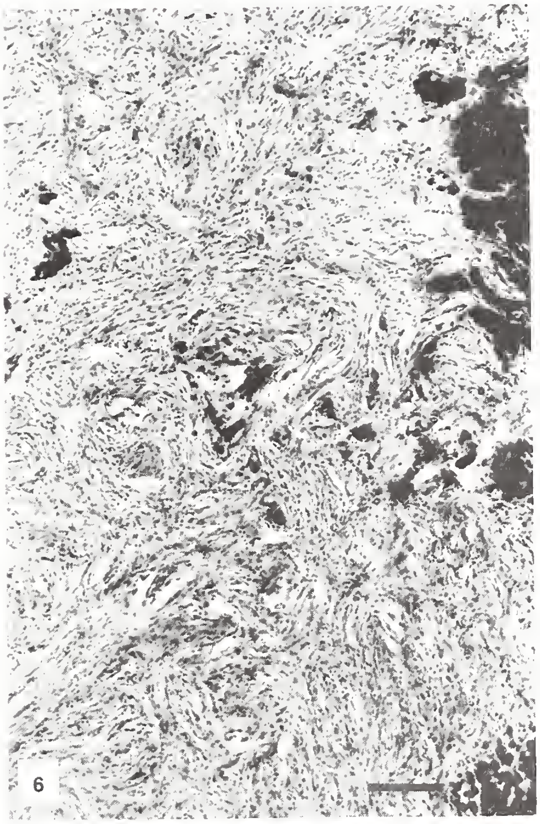


FIGURE 4.—Micrograph of a small melanotic lesion in wild nibe. Melanophores are proliferating generally parallel to the base. Bar = 50 μm. H & E. × 160

FIGURE 5.—Typical whorling pattern in a well-differentiated melanophoroma. Many melanophoroma cells had abundant melanin in the cytoplasm. Bar = 40 μm. H & E. × 250

FIGURE 6.—Portion of a chromatophoroma in which the cells have less well-differentiated characteristics. Cells are arranged in a star-burst pattern. Melanin granules, which differ in amount between cells are few. Bar = 40 μm. H & E. × 200

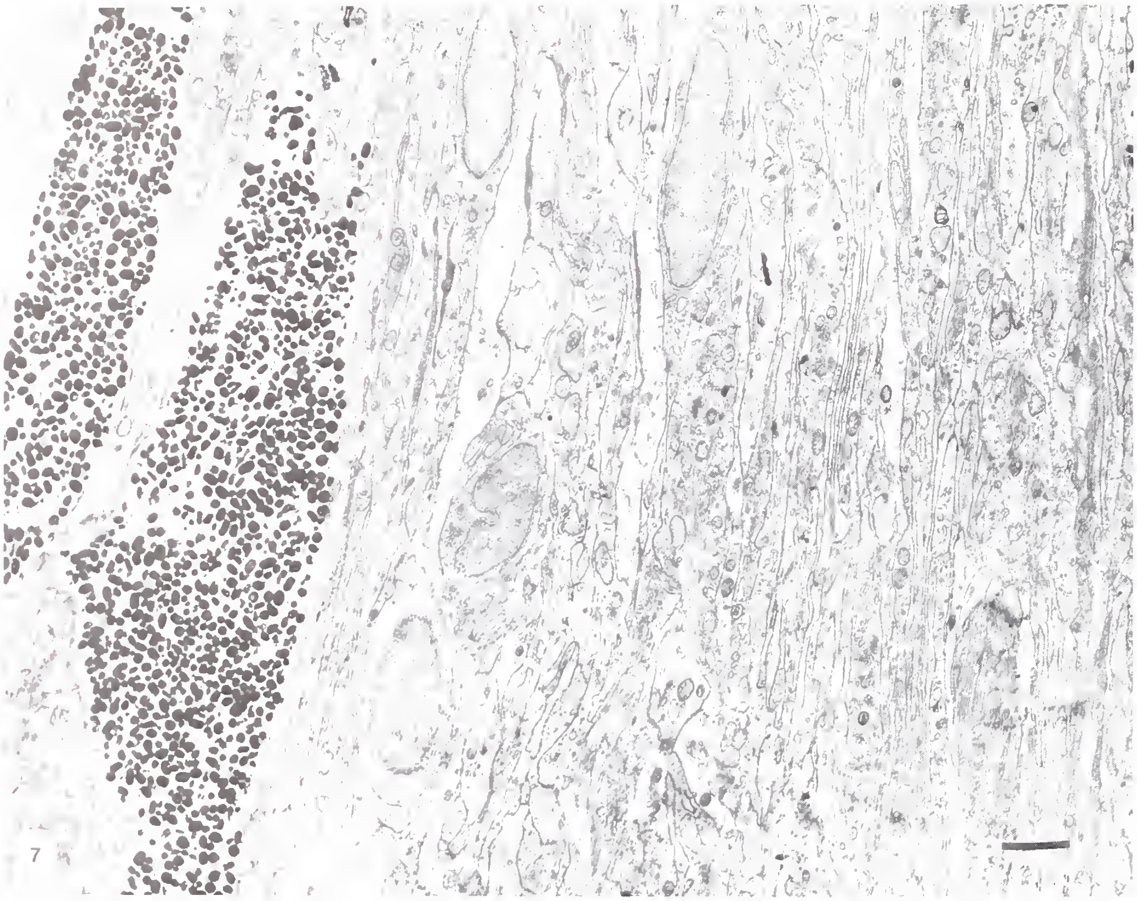


FIGURE 7.—Electron micrograph of a melanophoroma in nibe. Tumor cells have melanosomes in the cytoplasm and long thin cytoplasmic projections. Bar = 2 μm. × 4,500

stations I and H. However, the probability of catching nibe by rod and line from the shore tended to reduce the distance from the estuary at station I, and no nibe were caught near station H; nevertheless, a similar level of fishing effort was made. The fish at this latter station were accidentally caught by nets that were set; the incidence of the chromatophoroma was similar to that at station I. Therefore, we assume that the croaker of station I might incidentally migrate from this station to H. On the other hand, many rock bottom areas between stations I and K extend from the shore out to areas 30 to 40 m in depth; the rocky bottoms may be a limiting factor in the migration of adult nibe, although the eggs and larvae may be carried by the tide between these 2 stations because the river water at station I sometimes influences the water near stations H and K.

We examined the sex of the fish at station I in 11 surveys; the incidence was always higher in males than in females, and the males developed tumors earlier than did the females (table 2). The male-to-female ratio was approximately 10 to 11 in the total, but it was 10 to 9 in the fish smaller than 299 mm long and 10 to 18 in the fish larger than 300 mm, respectively. These results suggested that the tumor-bearing fish may not survive long. Because the sex was not always determined, only the body length of the fish was used as a parameter in our surveys.

Seasonal changes in the tumor incidences were observed at the stations I, Q, R, and S. For example, the incidence was low at station I from March to July (an average of 32%) and high in January and February (an average of 55%). However, the incidences were correlated to size of the fish caught, and no significant seasonal differences were observed when the fish of similar sizes were compared.

In light of these results, the incidences of the chromatophoromas in nibe at different sites are shown in a graph related to body length (fig. 11). The smallest tumor-bearing fish was 211 mm in total body length and was about 1.5 years old. The largest nibe caught during our surveys was 396 mm long and over 5 years of age. The incidences of tumors showed their tendency to increase with size of the fish at most sites, although the incidence at station I reached a plateau in the fish longer than 270 mm. Nibe inhabiting station I clearly had a uniquely high incidence of chromatophoromas.

Figure 12 shows the annual change of tumor incidence at station I; no significant differences were observed from 1973 to 1979. The incidence was significantly higher (75%) in 1980 than the 47% from 1973 to 1979 ($\chi^2 = 64$) and was reduced again to 37% in 1981. In 1980, the tumor incidence (an average of 91%) was especially high in small fish (240–269 mm in total body length). We have no evidence to explain why this incidence was so much higher in 1980.

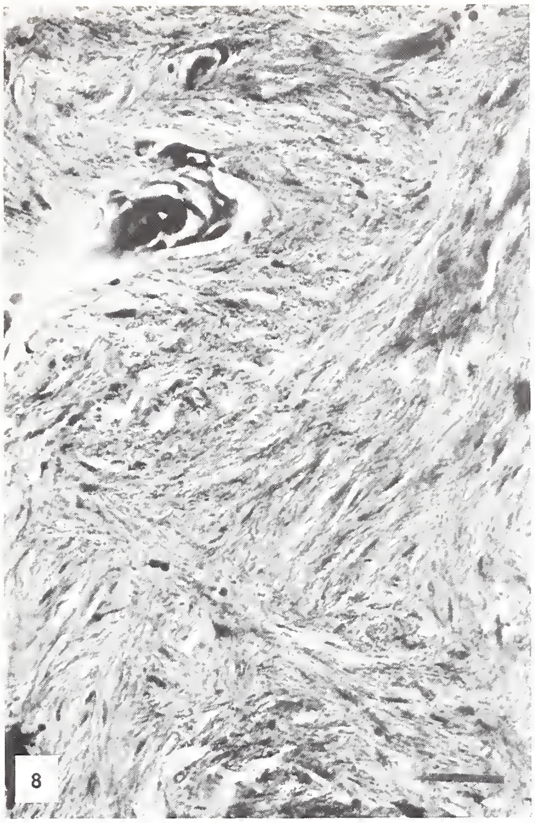


FIGURE 8.—Iridophore proliferation in a chromatophoroma. Nuclei and outline of the cells are unclear due to large number of guanine granules (reflecting platelets) in the cytoplasm. Bar=25 μm. H & E. × 500

However, the bottom of the harbor located at station I had been covered by sludge which was discharged from a pulp factory. This sludge, dredged in 1978 and 1979, contained potent mutagens, and thus we think the dredging might have influenced the increased tumor incidence in 1980.

Discussion

The above data describe the geographic distribution of the chromatophoromas in the croakers: nibe and koichi. The occurrence of tumors was seen sporadically in several coastal water areas off Honshu and Shikoku, but the incidence at station I was significantly higher than at the other stations. Therefore, we assumed that specific conditions exist at station I, either in the fish themselves or in their environment. To analyze the causes for this unique geographic distribution of tumorous fish, we conducted several studies and investigated genetic, viral, and chemical factors. An outline of the results obtained is as follows:

Local Population of Nibe

Taniguchi et al. (*9*) used several kinds of liver isozymes of nibe, such as alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, phosphoglucomutase, and glutamic-oxaloacetic transaminase as genetic markers because these isozymes show genetic variations controlled by 2 and 3 alleles. The phenotypes of these isozymes of nibe, which were caught at survey stations, were detected by means of electrophoresis, and the frequencies of the genotypes were calculated according to the formulas by Kimura (*10*) and Nei (*11, 12*). The results clearly showed that nibe distributed among the present survey stations were divided into the following genetic populations: Shizuoka, Mie-Wakayama, Kochi, and Miyazaki (*13*). However, no significant genetic differences were observed between nibe caught at stations I and K (Taniguchi N, et al: Unpublished data).

Examination for Viral Agents

A large number of tumorous tissues, in vitro cultured cells derived from the tumors, and in vitro cultured cells treated with 5-iododeoxyuridine were observed by electron microscopy. No virus-like particles were seen in any of these specimens. The assay by means of reverse transcriptase for detection of retroviruses was done by Ono et al. according to their method (*14*). These results were also negative.

Mutagenicity Testing and Chemical Analysis of Environmental Substances

The coast of the Kii Peninsula is generally clear, free of urban waste, and is sparsely populated. Large factories are also rare; however, 3 large pulp and paper factories are located near station I. Therefore, if certain chemicals are involved in the development of the neoplasm, they might originate from the industrial waste of these factories. For this reason, the discharged sewage and sediment were chemically analyzed by the Rec assay or the Ames test, or both. The amounts of substances regulated by law, such as polychlorinated biphenyls and heavy metals, were low in such samples. When a freeze-dried sample of the wastewater and ether extracts from the sediment were examined for their mutagenicity, both extracts originating from 1 of the 3 factories showed a positive reaction both in the Rec assay and the Ames test (*15*). In addition, the livers from the *N. mitsukurii,* which were caught at station I, contained Rec assay-positive substances (*16*). Similar factories also exist close to stations A, C, D, and X. Therefore, corresponding samples, wastewater, and sediment samples from the factories and nibe at stations A and D were examined by the same methods. All the samples at station D were negative in the Ames test and the Rec assay. The wastewater and sediment samples at station A showed weak DNA-damaging and mutagenic potency. The liver extracts of the fish at station A contained weak DNA-damaging substances. When Tomita, Kinae, and Kawane examined the mutagenic activity of seawater at 5 stations, including stations A, D, I (located close to factories) and stations C and K with no factories nearby, all the samples showed variable mutagenic activities. However, the yield (milligrams/10 liters of seawater) of organic compounds that could be extracted by diethylether and methanol was as shown below:

	Yield, mg/10 liters seawater by:	
Station	Diethylether	Methanol
I	10.0	208
A	9.1	236
D	4.4	193
C	66.0	173
K	3.9	170

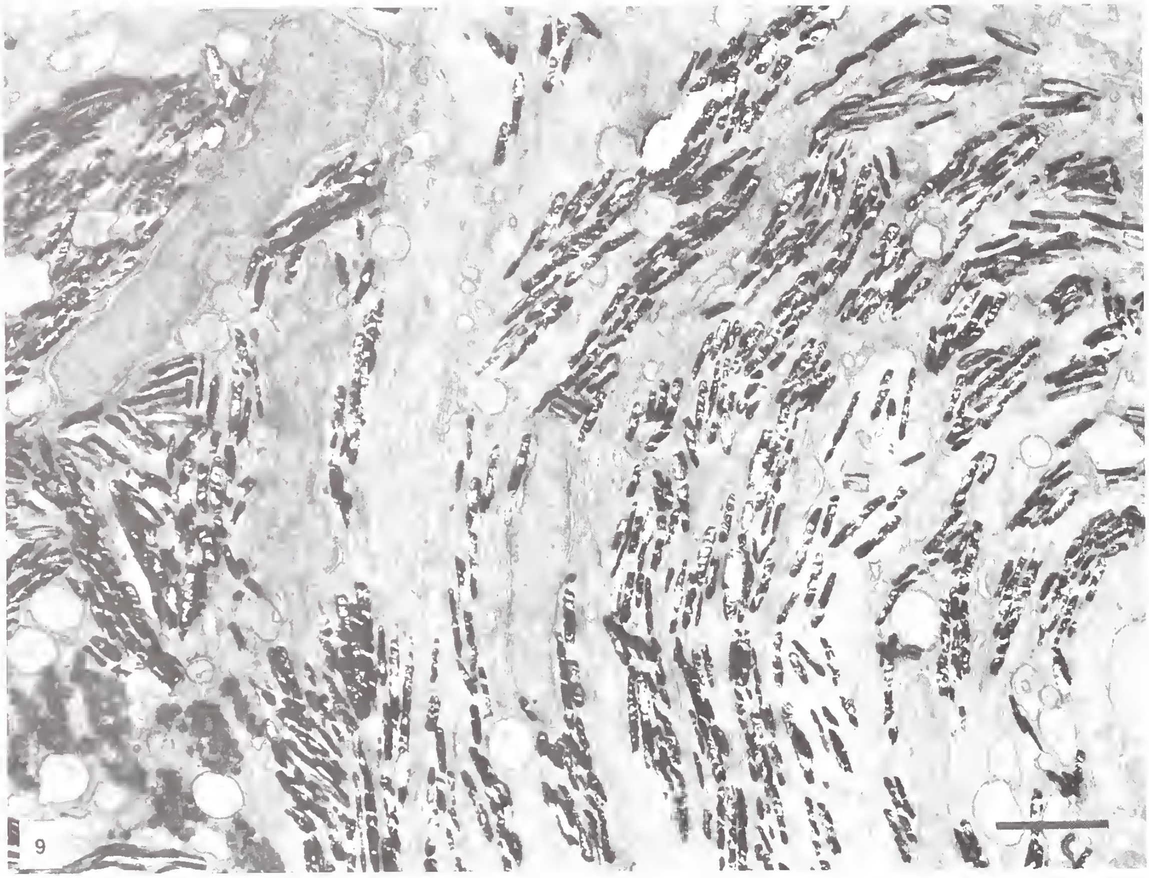


FIGURE 9.—Electron micrograph of an iridophoroma. Tumor cells contain reflecting platelets in the cytoplasm. Bar = 2 μm. × 7,540

The liver samples of the fish at station K showed neither mutagenic nor DNA-damaging activities (Kawane R: Unpublished observations).

Local Accumulation of a Catfish, Plotosus anguilars, *With Skin Melanosis*

If environmental chemicals are etiological factors in the development of pigment cell neoplasms in the croaker, the same substances might cause neoplasms or related disorders in other species of fish which inhabit the same area. Therefore, several species of fish caught at station I were examined for internal and external tumors. One sole, *Areliscus joynerii*, 1 black sea bream, *Mylis latus*, and 1 perch, *Lateolablax japonicus*, had adenomatous polyps in their stomachs. These tumors were found in large fish too few in number for epizootiological consideration. A species of catfish, *Plotosus anguilars*, was caught at 11 survey stations from 1979 to 1980, and the incidence of fish with unusual chromatophore hyperplasia was as follows:

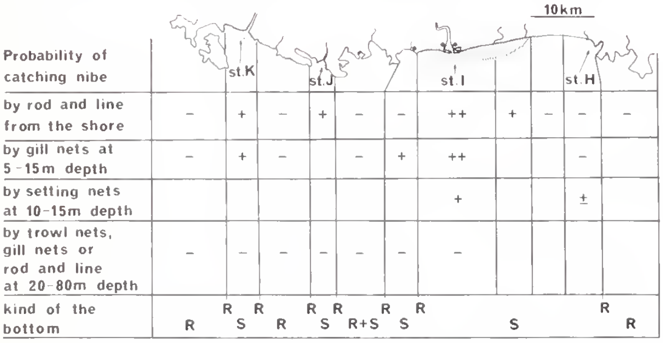

10km
Probability of catching nibe
st.K
st.J
st.I
st.H
by rod and line from the shore
– + – + – – ++ + – – –
by gill nets at 5-15m depth
– + – – + ++ –
by setting nets at 10-15m depth
+ ±
by trowl nets, gill nets or rod and line at 20-80m depth
– – – – – – –
kind of the bottom
R R R R R R R
R S R S R+S S S R

FIGURE 10.—Map of the coastal area between stations H and K showing the probabilities of catching nibe. ++ = nibe were caught most commonly; + = nibe were caught usually but the probability of catching them was less than one-fifth of ++; – = no nibe were caught; R = rock bottom areas from the shore to offshore of more than 20 m deep; S = sand bottom areas at the shore; solid circles = places where large pulp and paper factories are located.

Station	Incidence, %	No./total
I	13.5	15/111
K	4.8	10/210
R	2.5	1/40
D	0	0/19
H	″	0/36
J	″	0/10
L	″	0/43
M	″	0/44
Y	″	0/32
Toba	″	0/28
Kii-nagashima	″	0/123

when the fish larger than 150 mm in body length were designated as effective fish. The incidence at station I was significantly higher than that at station K ($\chi^2 = 6.57$).

TABLE 2.—*Sex difference of chromatophoroma incidence in nibe at station I*

Total body length, mm	Males			Females			Total		
	No. of fish:			No. of fish:			No. of fish:		
	Examined	Bearing tumors	Percent	Examined	Bearing tumors	Percent	Examined	Bearing tumors	Percent
210–239	37	8	21.6	35	1	2.8	72	9	12.5
240–269	91	49	53.8	83	22	26.5	174	71	40.8
270–299	173	96	55.5	155	66	42.6	328	162	49.4
300–329	89	52	58.4	155	64	41.3	144	116	47.5
≥330	3	1	33.3	18	9	50.0	21	10	47.6
Total	393	206	52.4	446	162	36.3	839	368	43.8

Figures 13 and 14 illustrate such a lesion, in which a black spot appears on the dorsal side of the head. The fish had 2 other lesions which appeared black and gray and were on the ventral portion. The pigmented lesions were slightly swollen in some fish. The largest one was 10 mm in diameter and 1.5 mm thick. They consisted of multilayered dendritic melanophores or iridophores which usually proliferated parallel to the base. The cells appeared to have lost the regulation of the color adaptation mechanism. In the adjacent normal skin, melanophores lay in a single layer under the basal lamina and had concentrated melanin granules at the central portion of the cells. On the other hand, the melanophores in the hyperplastic lesions had dispersed melanosomes in the cytoplasm. The histological patterns of the lesions were almost like those of early lesions in the croaker chromatophoromas, although we did not observe histological features which suggested the lesions were malignant in the catfish. It is uncertain whether the chromatophore hyperplasia of the catfish is neoplastic; however, it is remarkable that unusual chromatophore proliferations in the 2 species of fish accumulated at station I. The results suggest the role of chemical agents in the development of chromatophoromas in nibe at station I.

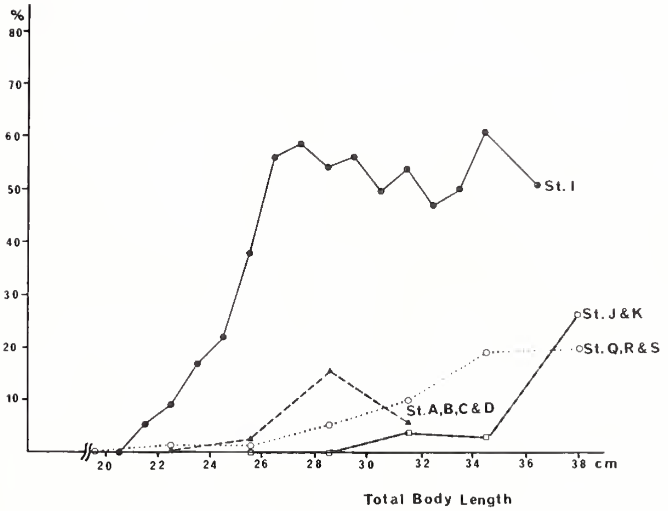


FIGURE 11.—Correlation between body size and chromatophoroma incidence in *N. mitsukurii* which were caught at several areas. Average tumor incidences are shown graphically.

CHEMICAL INDUCTION OF PIGMENTED LESIONS IN THE CROAKER NIBE (*N. mitsukurii*)

If environmental chemicals play a role in chromatophoroma development in nibe, the tumor may be induced by the administration of known carcinogens. Therefore, we exposed tank-reared nibe to DMBA, MNNG, or NP. We used the first 2 compounds because DMBA induces melanotic lesions in hamsters (*17*) and MNNG induces melanosis of the skin in rainbow trout when given during embryonic development (*18*); NP is a widely used antibacterial drug in fish culture (*19*). We initially used NP to prevent infectious diseases of the fish after handling during the first experimental procedures. However, the carcinogenic properties of NP were suggested during the course of the studies, and later we found that it is indeed a carcinogen in mice as well as in fish.

Induction of Pigmented Lesions: Experiment 1

Materials and Methods

The techniques for hatching and rearing nibe were established in 1978 by Taniguchi and a year later by Kumai.

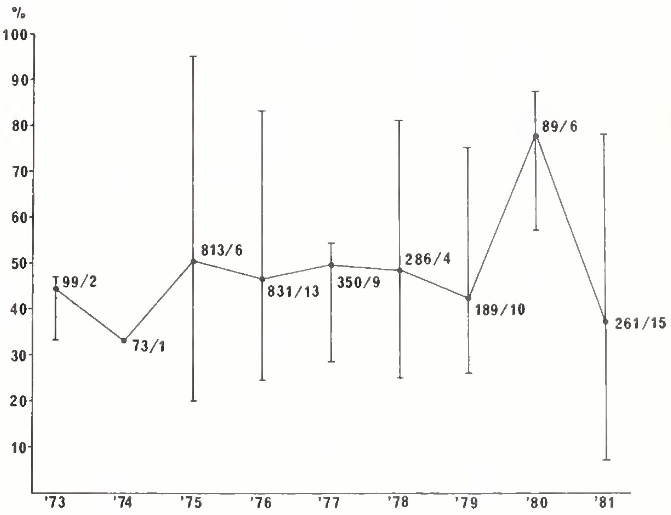
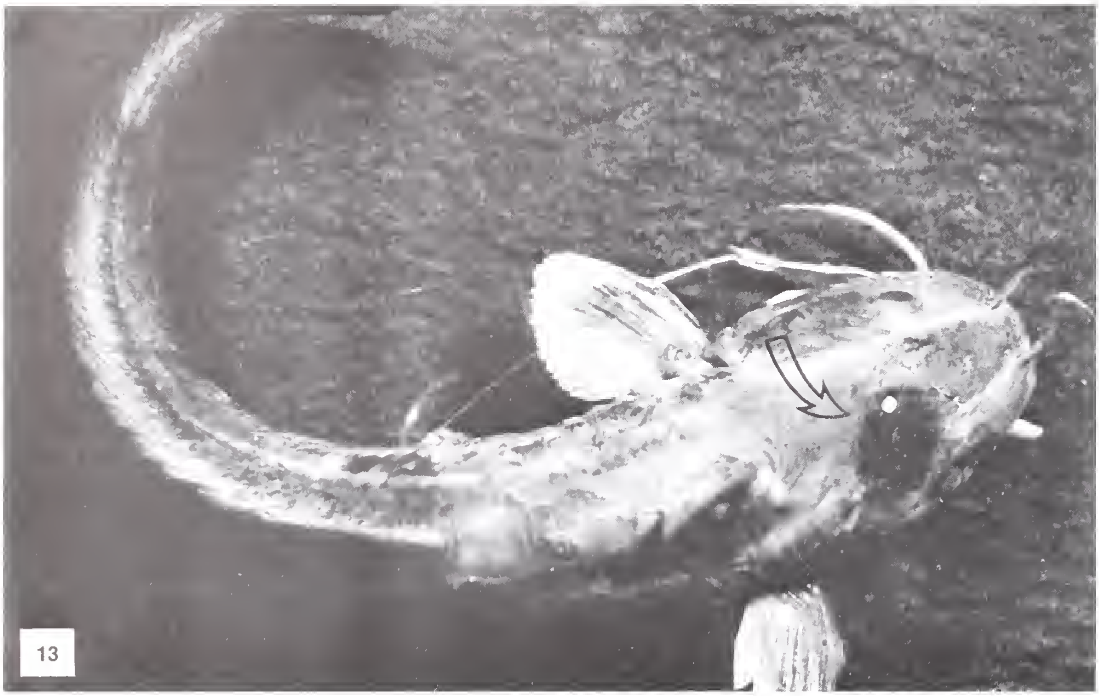


FIGURE 12.—Annual change of the chromatophoroma incidence in nibe at station I. Numerators of the fraction in the graph show the No. of effective fish, and the denominators show the times of the effective survey. *Vertical lines* indicate the highest and lowest incidences.



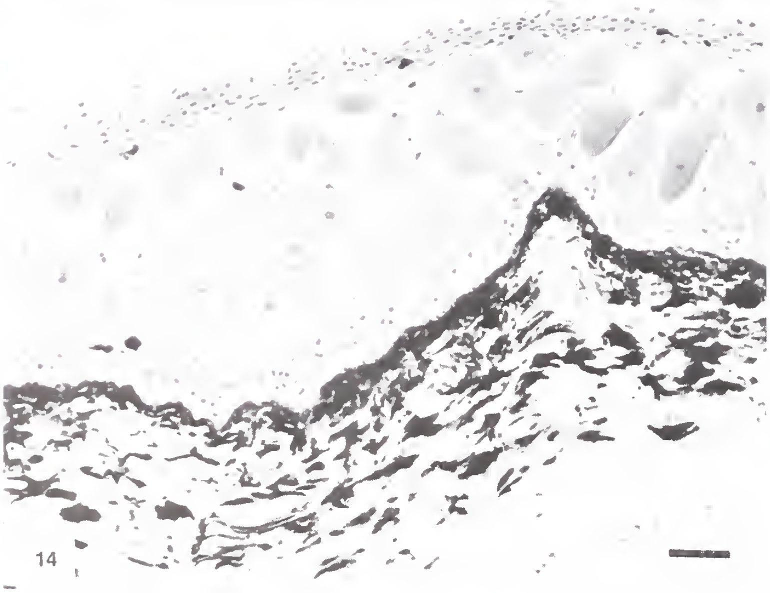


FIGURE 13.—A catfish, *Plotosus anguilars*, with skin melanosis (*arrow*) caught at station 1, had 2 other pigmented lesions under the jaw.

FIGURE 14.—Histological appearance of the lesion shown in figure 13. Bar = 20 μm. H & E. × 400

We used tank-reared 5-month-old nibe derived from the parental fish caught at station R during the spawning season in 1978. The fish, hatched in a tank in Kochi University in April, were divided into 4 groups. The average size of the fish was approximately 13 cm. Group 1: Fifty fish received a single sc injection of 1 to 2 mg of DMBA (Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.1 to 0.2 ml olive oil. Group 2: Fifty milligrams of MNNG (Aldrich Chemical Co., Milwaukee, Wisc.) was dissolved in 200 ml saline; 50 fish received a single gastric ingestion of approximately 100 mg MNNG/kg body weight. Group 3: Fifty fish received a single sc injection of 0.1 to 0.2 ml of olive oil and a single gastric ingestion of 0.2 to 0.3 ml of saline. Group 4: Approximately 300 nibe were held as untreated controls.

All the fish used in the experiment had been exposed to a solution of NP when they were moved from one tank to another. We exposed the fish of groups 1 to 3 again to

1 ppm of NP solution for 1 hour immediately after the treatment to prevent the occurrence of infectious disease after handling. Groups 1, 2, and 3 were kept in separate 1,000-liter tanks, and group 4 was kept in a 5,000-liter tank. Minced fishmeat was usually given as food. Surface observations were done on the living fish at 7 and at 13 months. Selected samples for necropsy were chosen from the respective groups at 8 months. At 18 months all the fish were killed and lesions on the skin and of the inner organs were histologically examined.

Results

Small melanotic lesions resembling pigmented nevi developed approximately 3 months after the initiation of the experiment, and the number of fish bearing the lesions increased with the experimental period. When examined at 7 months, the incidences were 73, 59, 23, and 13%, respectively, in groups 1, 2, 3, and untreated controls (table 3). The differences in the incidence between the DMBA and the olive oil–saline groups and between the MNNG and the olive oil–saline groups were significant ($\chi^2 = 10.4$ and 10.4, respectively). Because several fish were selected and necropsied at 8 months, the incidences in the remaining fish at 13 months could not be directly compared with those at 7 months but could be compared with those at 18 months; necropsies were not performed at 13 months. The total incidences in the groups of fish from which some were killed at 8 months and others observed at 13 months were 73% (8 of 11), 82% (28 of 34), and 42% (16 of 38), respectively, in groups 1, 2, and 3. The difference in the incidences became smaller with the experimental period because the incidence in the olive oil–saline group increased. The differences between the experimental groups and the control group were still significant at 13 months (see footnote *e* of table 3). However, at 18 months, the differences in the incidences among the groups were not significant.

The melanotic lesions occurred polycentrically on the skin on all portions of body including the head, trunk, tail, and fins. When the lesions were examined histologically at 8 months they showed a proliferation of pigment cells indistinguishable from the smallest lesions seen in the wild croaker. When examined 18 months later, the lesions in 13 fish showed features of typical chromatophoromas. All the fish given MNNG in the stomach had small polyps on the mucous membrane of the stomach, but no other kinds of tumorous lesions were observed in the fish.

Experiment 2

Materials and Methods

We repeated similar experiments in 1979 using station R stock. Tank-reared nibe at 5 months of age that had had no prior exposure to NP were divided into 5 groups: Group 1: One hundred fish received a single sc injection of 0.5–1.0 mg DMBA which was suspended in 0.1–0.2 ml of 10% lecithin–saline colloidal solution. Group 2: One hundred fish were immersed in seawater containing 20 ppm of MNNG for 1 hour. Group 3: Approximately 5 mg of methanol extract from the sediment at station I was suspended in 0.2 ml of 10% lecithin–saline and injected sc into the fish. Group 4: A single dose of 0.2 ml of the lecithin–saline solution was injected sc into the fish. However, all the fish of groups 1 to 4 were exposed to seawater containing 1 ppm NP for 1 hour after the treatment. Therefore, group 5 was kept as untreated fish without exposure to NP. The number of the fish in group 5 was indeterminate at the initiation of the experiment, and the fish examined at 8 months were randomly picked from a tank containing a large number of fish. These fish were kept according to the same methods described in experiment 1.

Results

Due to infectious diseases (groups 2 and 4) or to injury after injection (groups 1 and 3), a large number of the fish was lost by the third month. When the fish alive at 8 months were examined, the incidences of the lesions were as shown in table 3. Because the effective number of the fish in the lecithin–saline group was small, the reaction to the injection of DMBA or of the methanol extract was not clear in the experiment. The difference in the incidences between these 2 groups was not significant ($\chi^2 = 2.6$). The incidences were significantly different between the DMBA and the untreated groups ($\chi^2 = 11.8$) and between the MNNG and the untreated groups ($\chi^2 = 14.3$). The χ^2 between the methanol extract group and the untreated group was almost significant (3.2). All groups exposed to NP showed higher incidences than did the untreated controls, although the carcinogenic effect of the respective substances could not be clarified. These results of the experiment suggested again that NP might be carcinogenic to the fish.

Induction of Pigmented Lesions by Nifurpirinol: Experiment 3

Materials and Methods

Simultaneously with the beginning of experiment 2, a group initiated a series of experiments at Kinki University to clarify the effect of NP in the development of the pigmented lesions in nibe. Offspring from the parental fish caught at station I were used in the experiments, and granular furanase containing 10% NP was given (Dainippon Pharmaceutical Co. Ltd., Osaka) as follows: Nibe at 5 months of age that had been reared in a tank were divided into 6 groups and exposed to seawater containing 0, 0.5, 1, and 2 ppm NP for 1 hour, twice a day for 7 days. These groups were kept in 500-liter tanks. Because the untreated group of fish had their water supply cut off for 1 hour during the experiment, another 2 control groups were prepared as untreated groups (untreated group I) and approximately 204 fish (untreated group II). The untreated group II was kept in a 3,000-liter tank, and the other groups were kept in separate 500-liter tanks. The fish of untreated group II were used for other purposes 13 months later. The other fish were held for 20 months at which time the experiments were terminated. Surface examinations were done at 11 and 13 months, and histological examinations of the lesions were performed on the fish necropsied at 20 months.

TABLE 3.—*Induction of chromatophore hyperplasia or neoplasia in the croaker,* N. mitsukurii, *by DMBA or MNNG with exposure to NP at station R*

Expt No.	Initial No. of fish	Compound given and dosage[a]	Exposure route	Exposure to NP[b]	Period after initiation of expt, mo: 7		8		13		18	
					No. with lesion/No. examined	Percent	No. with lesion/No. examined	Percent	No. with lesion/No. examined	Percent	No. with lesion/No. examined	Percent
1	50	1–2 mg DMBA	sc injection	Twice	11/15	73	4/4[c]	100	4/7[d]	57	4/4	100
	50	2–3 mg MNNG	Gastric intubation	"	26/44	59	7/10[c]	70	21/24[d]	88	23/24	96
	50	Olive oil, 0.1–0.2 ml, and saline, 0.2–0.3 ml	Olive oil sc + saline by gastric intubation	"	9/40	23	6/10[c]	60	10/28[d]	36	26/28	93
	300	Untreated		Once	32/246	13	4/10[c,e]	40				
2	100	0.5–1 mg DMBA	sc injection	"			23/68	34				
	100	20 ppm MNNG	In water (1 hr)	"			3/4	75				
	100	Methanol extract, 5 mg in 0.2 ml lecithin–saline	sc injection	"			6/33	18				
	100	10% Lecithin–saline, 0.2 ml	sc injection	"			1/4	25				
		Untreated		None			1/38	2.6				

[a] *See* text for details.

[b] The fish were exposed to 1 ppm NP in water for 1 hr twice, once, or not at all.

[c] The fish were selected and necropsied for histological observation.

[d] Because several fish were selected and necropsied at 8 mo, we could not compare incidences directly with those at 7 mo. However, it is possible for one to calculate the minimum and maximum incidences of the respective groups at 13 mo that might have been observed if no fish were selected and necropsied at the 8th mo, on the supposition that the pigmented lesions do not regress. Calculated incidences are as follows: DMBA group: between 73 and 93%; MNNG group: between 63 and 93%; and the olive oil–saline group: between 40 and 55%. Minimum and maximum calculable incidences are obtained by the following expression: Let the No. of fish alive at 7 mo be A, No. of fish with pigmented lesions at 7 mo be B, No. of fish with lesions that were necropsied at 8 mo be C, No. of fish with lesions at 13 mo be D, No. of fish without lesions at 13 mo be E, and the greater value of B or C and D be F. The minimum calculable incidence= F/A, and the maximum calculable incidence=(A−E)/A.

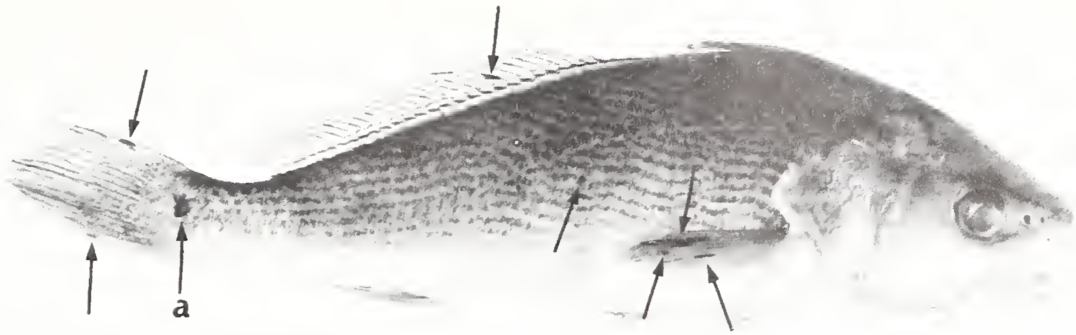
[e] Fish were used for other purposes 10 mo later.

Results

Most of the fish in the 2-ppm NP group died due to subacute toxicity of NP within 40 days (table 4). When examined at 11, 13, and 20 months, the 1- and the 0.5-ppm NP groups showed high incidences of pigmented lesions, whereas the incidences in the control groups were extremely low. Not only the percentage of the fish with pigmented lesions but also the number of lesions on individual fish showed a dose-response in the experiments. The average number of pigmented lesions per fish in the 1-ppm group was threefold or greater than that of the 0.5-ppm group. The incidences and numbers of lesions increased with the length of experimental period and reached 100% and 11.5 in the 1-ppm group and 71% and 4.0 in the 0.5-ppm group, respectively, at 20 months. The incidences of the 0-ppm group and of the untreated (I) group seem to be different; however, the difference was not significant ($\chi^2 = 2.3$). When the lesions were observed histologically at 20 months, most of them were similar to those of the smallest pigmented lesions in wild nibe. The development of typical chromatophoromas which showed whorling or star-burst patterns were observed in the fish at an incidence of 38% (6 fish) in the 1-ppm group, 29% (11 fish) in the 0.5-ppm group, 6% (2 fish) in the 0-ppm group, and 0% in the untreated (I) group. Representative features of such lesions are shown in figures 15–18.

Discussion of the Experimental Data

The results obtained by these experimental studies clearly show that nibe have a high susceptibility to the development of pigmented lesions when chemicals, such as DMBA, MNNG, and NP, were given. They also suggest that the nevus-like pigmented lesions probably are an early



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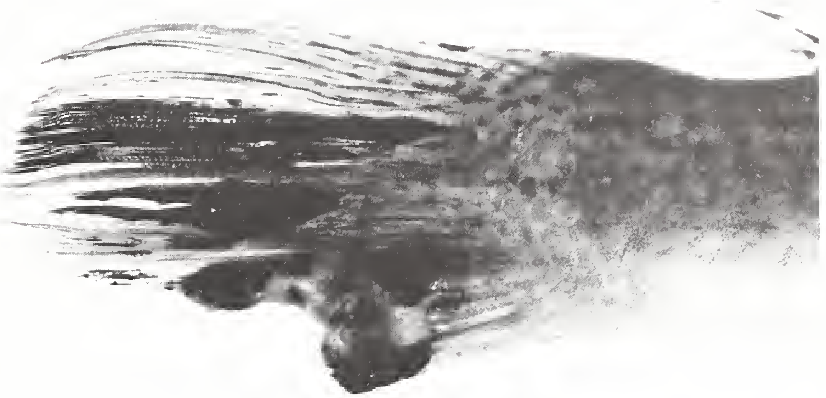

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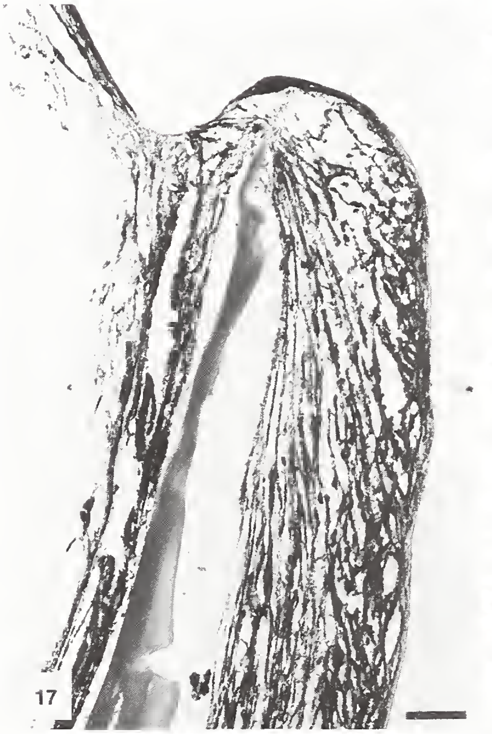

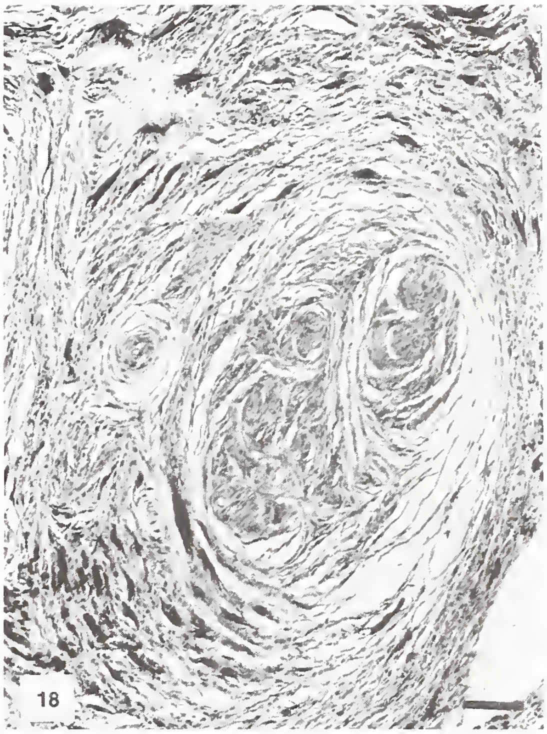

Figure 15.—Pigmented lesions (*arrows*) in tank-reared nibe at 11th mo after exposure to 1 ppm NP.

Figure 16.—Chromatophoromas which developed in nibe at 20th mo after exposure to 1 ppm NP.

Figure 17.—Histological appearance of pigmented lesion on scale of fish shown in figure 15 (*arrow* a). Bar = 40 μm. H & E. × 200

Figure 18.—Histological appearance of lesion in fish shown in figure 16. Bar = 40 μm. H & E. × 200

TABLE 4.—*Induction of proliferative pigmented lesions in nibe by NP at station I*[a]

Initial No. of fish	Dose of NP, ppm[b]	No. of fish with pigmented lesions/ average No. of lesions/fish					
		11 mo	Percent	13 mo	Percent	20 mo[c]	Percent
50	2	3/4 2.0	75	2/2 10.0	100		
50	1	23/37 1.8	62	20/23 7.0	87	16/16 11.5	100
52	0.5	22/52 0.5	42	36/49 1.9	73	27/38 4.0	71
100	0	0/39 0	0	2/38 0.2	5	5/32 0.8	16
50	Untreated group I	0/40 0	0	—		1/39 0.025	2.5
204	Untreated group II	—		6/204 0.034	2.9	—	

[a] *Dashes* indicate use of tank-reared nibe which were obtained from the parental fish caught at station I in this experiment.

[b] NP was administered fourteen times for 1 hr each time.

[c] All the fish were killed and examined histologically.

stage of the chromatophoromas. Little is known concerning the carcinogenic activity of NP; however, DMBA and MNNG are well-known carcinogens. Tonomura and Sasaki (*20*) described the adverse effect of NP on chromosome aberration and DNA repair synthesis in cultured human cells. Recently, some of us found that NP is not only a potent mutagen but also a carcinogen. When tested in 2 strains of *Escherichia coli* WP and *Salmonella typhimurium* TA 100 in the absence of the rat liver microsomal activation system, NP exerted a potent mutagenicity (*21*). The results of carcinogenicity testing of NP by Kimura et al. (*22*) are shown in table 5. When NP was given sc to ICR mice, it resulted in high incidences of local sarcomas and pulmonary tumors in both sexes and of liver cell tumors in the males after 4 or 10 months. When NP was given by stomach tube, it induced forestomach (papillomas and squamous carcinomas) and pulmonary tumors, respectively, in high incidences. In addition, sporadic tumors such as skin papillomas and leukemias occurred in a short latent period in the mice given NP; thus NP was carcinogenic in mice as well as in nibe. Moreover, nibe are highly susceptible to chromatophoroma development when they are exposed to various chemical carcinogens, such as DMBA, MNNG, and NP.

In addition to the data shown in table 5, we necropsied untreated ICR mice by 3-month intervals to examine the occurrences of spontaneous tumors. The incidences of lung tumors were 0% at 3 months (14 females and 12 males), 3.7% (1 of 27 females) and 3.8% (1 of 26 males) at 6 months, 7.9% (3 of 38 females) and 5.7% (2 of 35 males) at 9 months, 22% (7 of 32 females) and 20% (6 of 30 males) at 15 months, and 29% (10 of 35 females) and 28% (5 of 18 males) at 18 months of age, respectively. Liver cell tumors were observed only in 1 female and 1 male mouse killed at 18 months of age. Mammary cancer occurred in approximately 5% of the breeder females older than 11 months. One case each of salivary gland and ovarian tumor were observed among 17 mice that were kept alive longer than 19 months. However, no spontaneous occurrences of skin tumors, subcutaneous sarcomas, gastric tumors, and leukemias were found during the observation period until they were 21 months old. Therefore, we consider that all the tumor types shown in the table were induced by the administration of NP. The incidence of lung tumors in the males given 500 mg NP/kg body weight sc and autopsied 4 months later was significantly higher than that in the 6-month-old untreated mice ($\chi^2 = 9.8$) and of those 12 months old ($\chi^2 = 10.2$), although the difference in females was not significant. The lung tumor incidences in the other experimental groups were always significantly higher than those in the controls shown in table 5. The average numbers of the lung tumor nodules were threefold higher in the mice given 1,600 mg NP/kg body weight as in those mice (both sexes) given 400 mg. Such a dose-dependent tendency was not observed for liver tumors.

DISCUSSION

The studies reported herein show the geographic distribution of chromatophoromas in the croaker nibe (*N. mitsukurii*) and koichi (*N. albiflora*), which inhabit the coastal waters off Japan; the data for the latter species are insufficient. The sporadic occurrences of chromatophoromas in the croakers were observed in several areas, and the tumor incidence in nibe was high (47% on average) at a survey station located at a certain area on the shore of the Kii Peninsula.

To develop a better understanding of the causes of such a unique geographic distribution of the tumor, we investigated possible genetic, viral, and chemical factors. During the course of our studies, we found that treatment with chemicals, such as DMBA, MNNG, and NP, to nibe at 5 months of age resulted in the development of pigmented lesions 3 to 11 months after administration. Compared with the feral fish which developed the tumor at an age older than 18 months, the latent periods were extremely short in the experimental fish. Whereas DMBA and MNNG are potent carcinogens, the tumorigenic potential of NP, an antibacterial agent used to treat water in fish culture, was unknown before our studies. We found that NP induced pigmented lesions in the croaker and when tested in a bacterial system, it showed a potent mutagenicity (*20*). In addition, when NP was given to mice, it induced several types of tumors with high incidences. On the basis of these and other results, we consider it likely that the chromatophoromas in the feral fish are induced by environmental chemicals, although we have not identified the specific ones.

However, we can affirm that tank-reared nibe are important and useful experimental animals in studies of fish chromatophoromas and as bioassay animals in carcinogenicity testing.

TABLE 5.—*Tumor incidences in ICR mice given NP*

Route of administration	NP dose, mg/g body wt	No. of exposures	Total dosage/kg body wt, mg	Length of expt, mo	Sex	No. of mice examined	Tumors								
							Lung			Fore-stomach		Liver		Other	
							No. of mice	Per-cent	No. of tumors/mouse examined	No.	Per-cent	No.	Per-cent	Site/type	No.
sc injection[a]	0.1	5	500	4	F	6	2	33	0.5	0		0		Skin	1
					M	6	4	67	0.8	0		0			
	0.05	8	400	10	F	28	22	79	2.1	1	4	1	4		
					M	17	12	71	1.0	0		9	53	Leukemia	1
	0.2	8	1,600	10	F	15	14	93	6.0	0		2	13	Local sarcoma and skin	1 1
					M	13	12	92	3.5	1	8	7	54	Local sarcoma	1
	0.2	24	4,800	3–10[b]	F	25	21	84	2.7	2	8	3	12	Local sarcoma and skin	18 1
					M	27	25	93	3.2	2	7	12	44	Local sarcoma and leukemia	21 1
	0	24	0	10	F	20	3	15	0.2	0		0			
					M	20	3	15	0.2	0		0			
Gastric ingestion[c]	1.0	12	12,000	12	F	18	15	83	3.3	17	94	1	6	Skin	1
					M	23	16	70	1.5	17	74	1	4		
Untreated control		0	0	12	F	78	12	15	0.2	0		0			
					M	78	8	10	0.1	0		0			

[a] The NP was given at 3-day intervals from birth.

[b] Twenty females and 22 males were necropsied at the 3d to 9th mo, with most at the 6th mo due to the development of local sarcomas. Incidences of lung tumors in the mice necropsied at the 10th month were 100% for both males and females. The average numbers of tumor nodules seen on the lung surface were 4.2 in females and 7.0 in males.

[c] The NP was given once a week from 4 wk of age.

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Preliminary Studies on the Usefulness of Medaka, *Oryzias latipes*, Embryos in Carcinogenicity Testing[1]

James E. Klaunig, Bruce A. Barut, *and* Peter J. Goldblatt[2]

ABSTRACT—Medaka (*Oryzias latipes*) embryos were exposed continuously for 10 days to diethylnitrosamine (DENA) at concentrations of 25, 50, or 100 ppm. Following exposure and after hatching, the embryos were placed in clean, carcinogen-free water. Fish were sampled for pathological examination after 1 month, 3, and 6 months. Grossly visible liver tumors were evident after 3 months in 10 and 30% of the fish treated with 50 and 100 ppm DENA, respectively. Following 6 months exposure, 4% of the fish treated with 25 ppm, 15% of those given 50 ppm, and 43% of those treated with 100 ppm DENA contained liver tumors. Focal areas consisting of 10 to 40 highly basophilic cells in the liver were noted at all the exposure concentrations. The incidence of the focal areas increased proportionately with the concentration of DENA and the age of the fish. Liver tumors were examined by light and electron microscopy. Most of the hepatic tumors were moderate to well-differentiated trabecular hepatomas, although 2 cholangiomas and 2 poorly differentiated hepatomas were noted. No liver lesions or tumors were observed in controls. — Natl Cancer Inst Monogr 65: 155-161, 1984.

Carcinogenic compounds have produced tumors in several small teleosts, making these species potential subjects for a carcinogen bioassay (*1–3*). The medaka (*Oryzias latipes*) is one such small fish that offers several advantages as a short-term in vivo model for carcinogen testing. Adult medaka are small (2 to 4 cm long), hardy, and are easily maintained in ordinary glass aquaria with minimal daily care; they can survive a variety of temperatures (from 5° to 35° C). Medaka are readily bred in aquaria all year by adjustments of the photoperiod and the water temperature. Each female fish can produce up to 30 eggs daily. These eggs (1.0–1.5 mm in diameter) are easily collected and handled. The fertilized eggs hatch within 11–12 days (at 25° C). Adult medaka have developed hepatic tumors after exposure to DENA (*3*) and MAM acetate (*4*). Ishikawa et al. (*3*) noted a 100% incidence of liver tumors in medaka exposed to 45 ppm DENA for 8 weeks. Aoki and Matsudaira (*4*) reported the induction of hepatic tumors in 9 of 12 fish 90 days following a single 24-hour exposure to 2.0 ppm MAM acetate.

Wales et al. (*5*) found hepatic tumors in adult rainbow trout 10 to 12 months following a 1-hour exposure of trout embryos to 0.5 ppm aflatoxin B_1. More recently, Hendricks et al. (*6*), using the embryo exposure model, noted the induction of both liver and kidney tumors in rainbow trout after a 1-hour exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

We initiated this study to investigate the effect of carcinogen treatment of medaka embryos on subsequent tumor development.

MATERIALS AND METHODS

Animals.—Sixty female and 40 male adult medaka [(*O. latipes*); Carolina Biological Supply Co., Burlington, N.C.] were placed together in a 15-gallon glass aquarium containing dechlorinated Toledo City water. Medaka were readily bred in the aquarium by adjustments of the photoperiod (16 hr light/8 hr dark) and the water temperature (28° C during the light period and 25° C during the dark). Approximately 1 hour after the light came on each day, the female laid her eggs and the males fertilized them. Each female fish produced 10–30 eggs daily. The eggs (1.0–1.5 mm in diameter) remained attached to the female medaka for several hours and were easily collected soon after spawning by removal of the female fish from the aquarium with a dip net and gently removing the egg cluster with watchmakers' forceps. The eggs from several females were pooled in a 100×20-mm petri dish containing sterile embryo rearing solution according to the formula shown in table 1 (*7*). A small amount of methylene blue (0.001%; U.S.P) was added to the solution. This dye stained dead and unfertilized embryos blue, whereas the viable fertilized embryos remained clear. Methylene blue was omitted from the embryo rearing solution used in the carcinogenicity studies. All adult fish (1 mo and older) were fed TetraMin Staple Food (Tetra Werke, Melle, Federal Republic of Germany) twice daily and freshly hatched brine shrimp three times weekly. Newly hatched fry received Liquifry Dried Plankton (Liquifry Company, Dorking, England) in the water once daily.

Preliminary toxicity studies.—Initial studies focused on the determination of the toxicity of DENA to medaka embryos. For exposure, medaka eggs were placed in plastic 24-well, multiplate culture dishes.

Each well was filled with 2 ml of embryo rearing solution

ABBREVIATIONS: DENA = diethylnitrosamine; MAM = methylazoxymethanol.

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[3] The authors thank Mr. Steven Knipe for his technical assistance and Mrs. Rose Kenney for her secretarial help.

TABLE 1.—*Composition of embryo rearing solution*[a]

Compounds[b]	Percent	Amount, ml
NaCl	15	1
KCl	0.3	1
$CaCl_2 \cdot 2H_2O$	0.4	1
$MgSO_4 \cdot 7H_2O$	1.63	1
H_2O distilled		96

[a] *See* (*7*).

[b] Compounds (sodium, potassium, and calcium chloride and magnesium sulfate) are given on the basis of weight/volume.

containing methylene blue, and a single viable, fertilized embryo was placed in each well. Embryos were treated with 1 of 11 concentrations of DENA ranging from 0 to 1,000 ppm. One hundred pooled embryos were treated at each DENA concentration. Each embryo was treated with the carcinogen dissolved in dimethyl sulfoxide from 4 hours postfertilization to day 10 of embryogenesis. Embryo rearing solution was changed after the tenth day of exposure, and the embryos were allowed to hatch. The time of hatching and survival of the embryos at hatching was recorded for each concentration of DENA.

Carcinogenicity experiments.—Based upon these preliminary toxicity studies, we treated the medaka embryos with 1 of 3 concentrations of DENA during embryogenesis and examined them for tumor formation up to 6 months after hatching. Fifty embryos were continuously treated with either 25, 50, or 100 ppm of DENA from 4 hours until 10 days after fertilization. Embryos were exposed individually in the multiwell plates as described above. After hatching, fry from the same concentration group were removed from the multiwell dishes and placed in a 4-liter glass aquarium. Fish were fed twice daily and the water was changed twice monthly. Ten fish were randomly removed from each glass aquarium for histopathological examination after 1 month and 3 months posthatching. After 6 months, the remaining survivors were removed. Dead or dying fish, or both, were removed from aquaria twice daily and examined for pathological changes. For histological sampling, fish were removed from the aquarium and anesthetized in a 1:4,000 solution of tricaine methanesulfonate. Fish were then fixed (totally immersed) in a 10% phosphate-buffered formalin solution. An incision made in the abdominal wall of each fish allowed the penetration of fixative into the visceral organs. After fixation for 48 hours, whole fish were processed for histology. Fish were dehydrated in ascending concentrations of ethanol followed by infiltration in paraffin. Each fish was mounted into a single paraffin block. A series of longitudinal paraffin sections were taken from each fish, which allowed the sampling of all major organs on the same slide. Sections were stained with hematoxylin and eosin, viewed, and photographed on a Zeiss photomicroscope.

TABLE 2.—*Preliminary toxicity of DENA on medaka embryo*[a]

Concentration of DENA, ppm	Percent survival at hatching
0	100
1	100
5	100
10	97
25	95
50	94
100	87
200	75
250	70
500	10
1,000	0

[a] One hundred fish were exposed (individually) in 2 ml embryo rearing solution from 4 hr to 10 days postfertilization.

Samples of grossly visible tumors were also taken for analysis by electron microscopy. A small (1-2 mm^3) piece of the tumor was removed before fixation in 10% formalin and fixed in 3% cacodylate buffer glutaraldehyde. After 4 hours of fixation, the tissue was rinsed in buffer, postfixed in 1% osmium tetroxide, dehydrated in ascending concentrations of ethanol, and embedded in Epon 812. Thin (60-80 mm) sections were cut from each tumor on an LKB III ultratome, collected on copper grids, and stained with uranyl acetate and lead citrate. Sections were viewed and photographed on a Zeiss 9S electron microscope.

RESULTS

Toxicity Studies

In the preliminary toxicity studies, the number of surviving hatched embryos after 10 days exposure to DENA was determined for each concentration (table 2). Concentrations of 1 and 5 ppm DENA displayed no effect on embryo survival and hatchability. Increasing the concentrations by 10 ppm and greater resulted in a progressive decrease in embryo survival. At 1,000 ppm DENA, no embryos survived the exposure. The majority of deaths at all concentrations occurred within the first 2 to 4 days of DENA treatment. At concentrations of 200, 250, and 500 ppm, the time of hatching for the surviving embryos was lengthened up to 21 days from the average of 12 days seen in the controls. Concentrations of 1 to 100 ppm DENA had no effect on embryo hatching time.

Carcinogenicity Studies

The 50 embryos treated with the 3 concentrations of DENA (25, 50, and 100 ppm) for the carcinogenicity experiments displayed a similar dose response to the carcinogen that was observed in the preliminary toxicity studies. After exposure and hatching of 50 embryos, 47, 47, and 42 embryos survived the 25, 50, and 100 ppm DENA treatment, respectively. Following the hatching and placement of the treated embryos into the aquaria, 18 subsequent deaths of fish occurred. Eight fish from the 100-, 7 from the 50-, and 3 from the 25-ppm treatment groups died. The 18 deaths occurred within 2 weeks after hatching. Necropsy and histopathological analysis of these fish failed to reveal the cause of death.

TABLE 3.—*Summary of medaka egg exposure to DENA*[a]

DENA treatment, ppm	No. of tumors at time after hatching, mo			Total
	1	3	6	
100	0/10	3/10	6/14	9/34
50	0/10	1/10	3/20	4/40
25	0/10	0/10	1/24	1/44
Control	0/10	0/10	0/30	0/50

[a] Fifty eggs were treated with the indicated concentrations of DENA continuously from fertilization until hatching. At hatching, fry were placed in clean dechlorinated water (no carcinogen added). Ten fish were sampled for each group after 1 mo and 3 mo. The remaining fish were sampled after 6 mo exposure.

Pathologic Findings

Ten medaka from each of the 3 embryo treatment groups were sampled after 1 month, 3, and 6 months posthatching for gross pathological and histopathological analysis. Appropriate controls were similarly sampled.

Gross Pathologic Findings

No pathology was noted in any of the sampled control fish. At the 1-month sampling time, no visible gross pathological lesions were evident in fish from any of the 3 exposure concentrations (table 3). After 3 months, 3 of 10 fish (30%) of the 100-ppm treatment group and 1 of 10 fish (10%) of the 50-ppm group possessed grayish-white nodules on their livers. The nodules occupied 25 to 50% of the total liver mass. Nodule-containing livers were approximately twice the size of livers from fish that did not contain the nodules. No hepatic nodules were observed in fish from the 25-ppm treatment group after 3 months. In the fish sampled at 6 months, 1 of 24 (4%) from the 25-ppm, 3 of 20 (15%) from the 50-ppm, and 6 of 14 fish (43%) from the 100-ppm treatment groups contained liver nodules. No other gross pathologic lesions were observed in any of the sampled experimental fish.

Histologic Findings

Histopathological evaluation of the treated and control fish revealed the liver to be the only organ pathologically affected by the exposure to DENA. Therefore, descriptions of light and electron microscopy in this study will be limited to hepatic pathology.

Control Liver

Light microscopic analysis of the livers of controls at the 3 sampling periods revealed a morphology consistent with that described previously by Ishikawa et al. (*3*) and Aoki and Matsudaira (*4*). The hepatic tissue consisted of sheets of parenchymal cells separated by blood sinusoids (fig. 1). No distinct lobular morphology was evident. Portal triads were generally absent with blood vessels and bile ducts distributed throughout the liver. Hepatocytes were usually polyhedral and contained a single oval nucleus surrounded by a granular cytoplasm. Large, clear areas of cytoplasm were frequently present. These regions stained positive with periodic acid-Schiff and were judged to contain glycogen.

Treated Liver

One Month

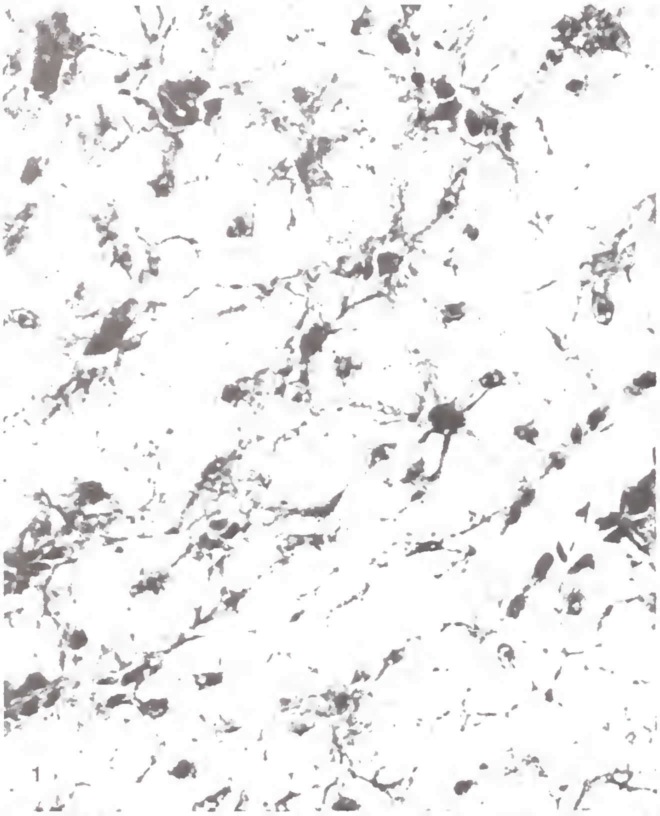
No liver changes were observed in the 25-ppm treatment group at the 1-month sampling time. In 3 of 10 fish from the 100-ppm group and in 1 of 10 fish from the 50-ppm group, focal areas of cellular alteration were noted in the liver. These focal areas consisted of 10–40 tightly packed, highly basophilic cells (fig. 2). Several of the examined focal lesions contained mitotic figures (1–2/focus). The cells within these focal areas were smaller than the surrounding normal appearing hepatocytes. An average of 2–3 focal lesions were observed in each examined liver section from each fish.

Three Months

At the 3-month sampling period, 2 of 10 fish from the 25-ppm, 6 of 10 fish from the 50-ppm, and 7 of 10 fish from the 100-ppm treatment groups displayed focal areas of highly basophilic cells in their livers. Three of these 7 fish from the 100-ppm group and 1 of the 6 from the 50-ppm group also contained hepatic tumors. The tumors appeared as typical trabecular hepatomas and were frequently separated into oval nodules by strands of normal appearing hepatocytes (fig. 3). The hepatic muralia in the tumors were up to 10 basophilic cells in thickness and were separated by blood sinusoids. Occasional mitotic figures were seen in the tumor. No fibrosis or cirrhosis was noted, although small areas of necrosis were a frequent finding in the tumors. The tumor nodules frequently appeared well circumscribed by the normal appearing hepatocytes. No invasion of surrounding nonhepatic tissue or distant metastases to other organs were observed.

Six Months

The remaining 24 fish from the 25-ppm treatment group were sampled after 6 months. Three of the fish had multiple areas of focal alteration in their livers; in 1, a trabecular tumor was found. The tumor cells were arranged in thickened muralia, and the tumor was encircled by a rim of normal-appearing hepatocytes. Three of the 20 remaining fish from the 50-ppm group displayed multiple nodular trabecular tumors in the liver after 6 months. Focal areas of alteration were seen in 10 of these fish, including the 3 that contained tumors. Fish from the 100-ppm exposure group displayed areas of cellular alteration in 11 of the remaining 14 survivors. These focal lesions were multiple and found throughout the liver. Tumors were also seen in 6; these for the most part were of a trabecular morphology. However, two variations were noted. Poorly differentiated tumors containing spindle-shaped cells in a whorllike pattern were evident in 2 of the tumor-bearing fish. The spindle-shaped cells were pleomorphic and stained poorly with hematoxylin and eosin. The cholangiomas found in 2 of the 6 tumor-bearing fish (fig. 4) were characterized by proliferating bile duct cells. Frequently, enlarged ducts had a cystlike appearance with amorphous eosinophilic debris within the



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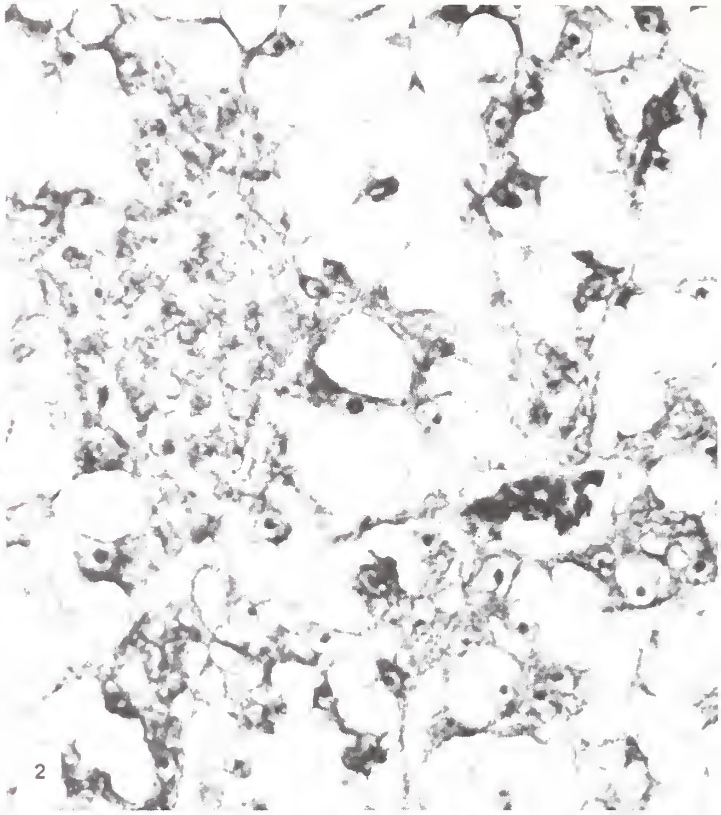

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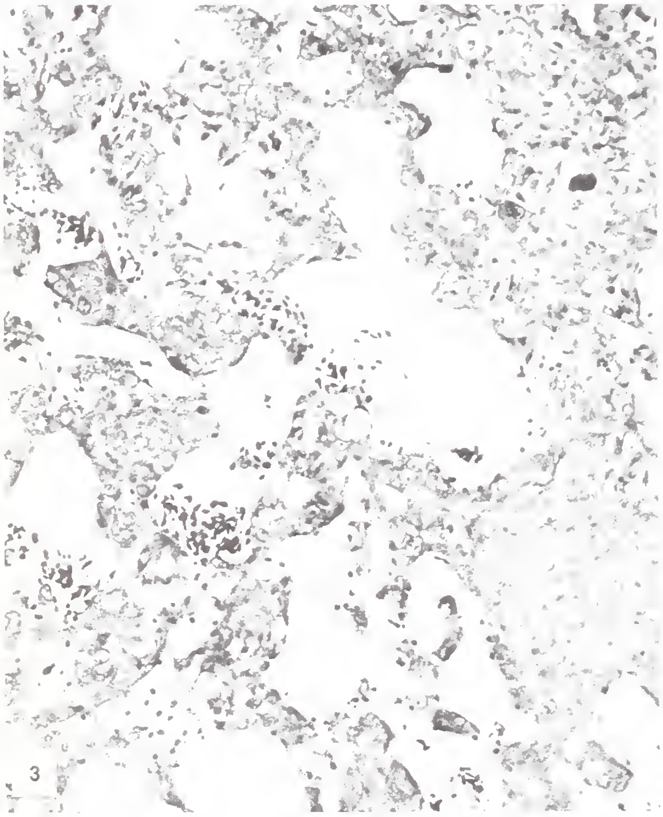

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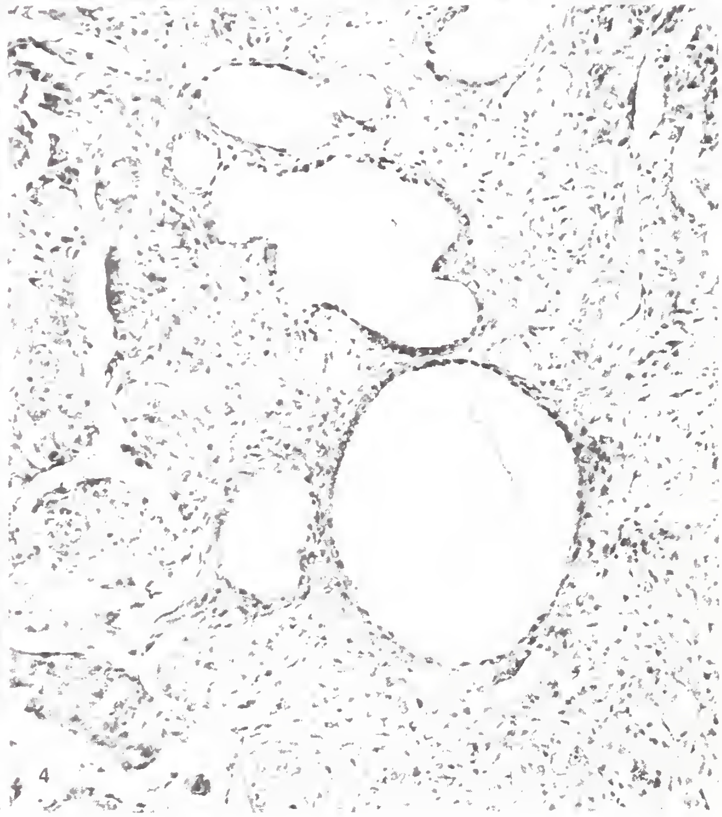

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FIGURE 1.—Light photomicrograph of control medaka liver sampled at 6 mo posthatching. No distinct lobular morphology was evident. Hepatocytes were usually polyhedral and contained a single nucleus and large areas of glycogen. × 400

FIGURE 2.—Light photomicrograph of liver from 100-ppm DENA-treated medaka liver at 3 mo posthatching. A focal area of packed, highly basophilic cells is evident. × 400

FIGURE 3.—Light photomicrograph of a trabecular hepatic tumor from a 100-ppm DENA-treated medaka 6 mo posthatching. × 125

FIGURE 4.—Light photomicrograph of a cholangioma from a 100-ppm DENA-treated medaka liver 6 mo posthatching. × 125

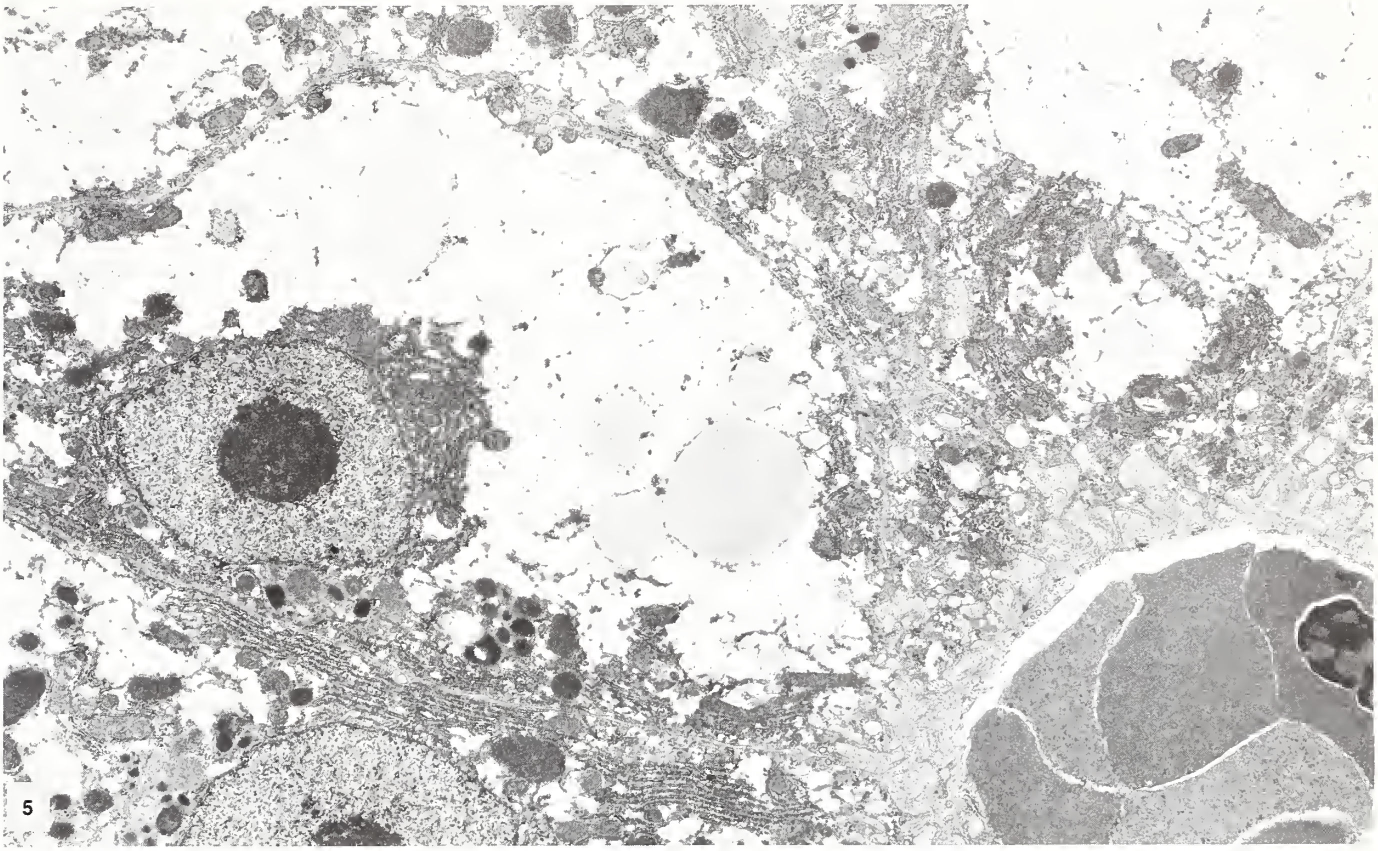

FIGURE 5.—Electron micrograph of a control medaka liver sampled 6 mo posthatching. Several hepatocytes are evident bordering a blood sinusoid. Hepatocytes contain a single, oval nucleus surrounded by multiple profiles of paralleled stacked rough endoplasmic reticulum and mitochondria. Large portion of the cytoplasm is occupied by glycogen and lipid droplets. × 3,000

lumen of the cyst. The 2 poorly differentiated tumors and 2 cholangiomas were in fish livers that also contained trabecular tumors; however, these lesions were not in the same liver. No metastatic growth or invasion of adjacent organs was found in any of the fish.

Electron Microscopic Findings

Liver tumors from the 50- and 100-ppm DENA-treated fish at the 6-month sampling period were examined by transmission electron microscopy. Livers from controls were also sampled for study by electron microscopy after 6 months.

A typical hepatocyte from a control contained a single oval-shaped nucleus. Usually, a single prominent nucleolus was evident centrally located within the nucleus (fig. 5). The cytoplasm contained numerous cellular organelles including parallel stacks of rough endoplasmic reticulum, Golgi, oval-shaped mitochondria, and lysosomes. Large areas of cytoplasm were typically filled with glycogen. Generally, 4 to 10 lipid droplets of varying size appeared within this glycogen-rich cytoplasmic region. Tight junctional complexes were evident between adjacent hepatocytes.

Tumors sampled for electron microscopy were classified as moderate to well-differentiated trabecular hepatocellular carcinomas. Cells in the tumors appeared smaller than control hepatocytes. Nuclei were more elongated and sometimes contained pseudoinclusions of cytoplasm. The nucleoplasm was frequently less electron dense than that seen in control nuclei. Multiple nucleoli were occasionally observed. The cytoplasm of the tumor cells usually contained numerous strands of rough endoplasmic reticulum (fig. 6) that were arranged in parallel stacks. They frequently surrounded other cytoplasmic organelles, such as mitochondria and lysosomes. Occasionally, these strands formed large circular whorl patterns within the cytoplasm. These whorls contained approximately 30 strands of rough endoplasmic reticulum tightly packed in concentric rings. In contrast to the control hepatocytes, cells within the trabecular tumors displayed little glycogen within their cytoplasms. A qualitative decrease in the number of mitochondria in the tumor cells from that of control hepatocytes was also apparent. Intracellular and extracellular vacuoles were frequently evident in the tumor cells. An occasional finding within the tumor tissue was the presence of lipid-laden cells (fig. 7). Large numbers of lipid droplets were present in both the cytoplasm and nuclei of these lipid-laden cells.

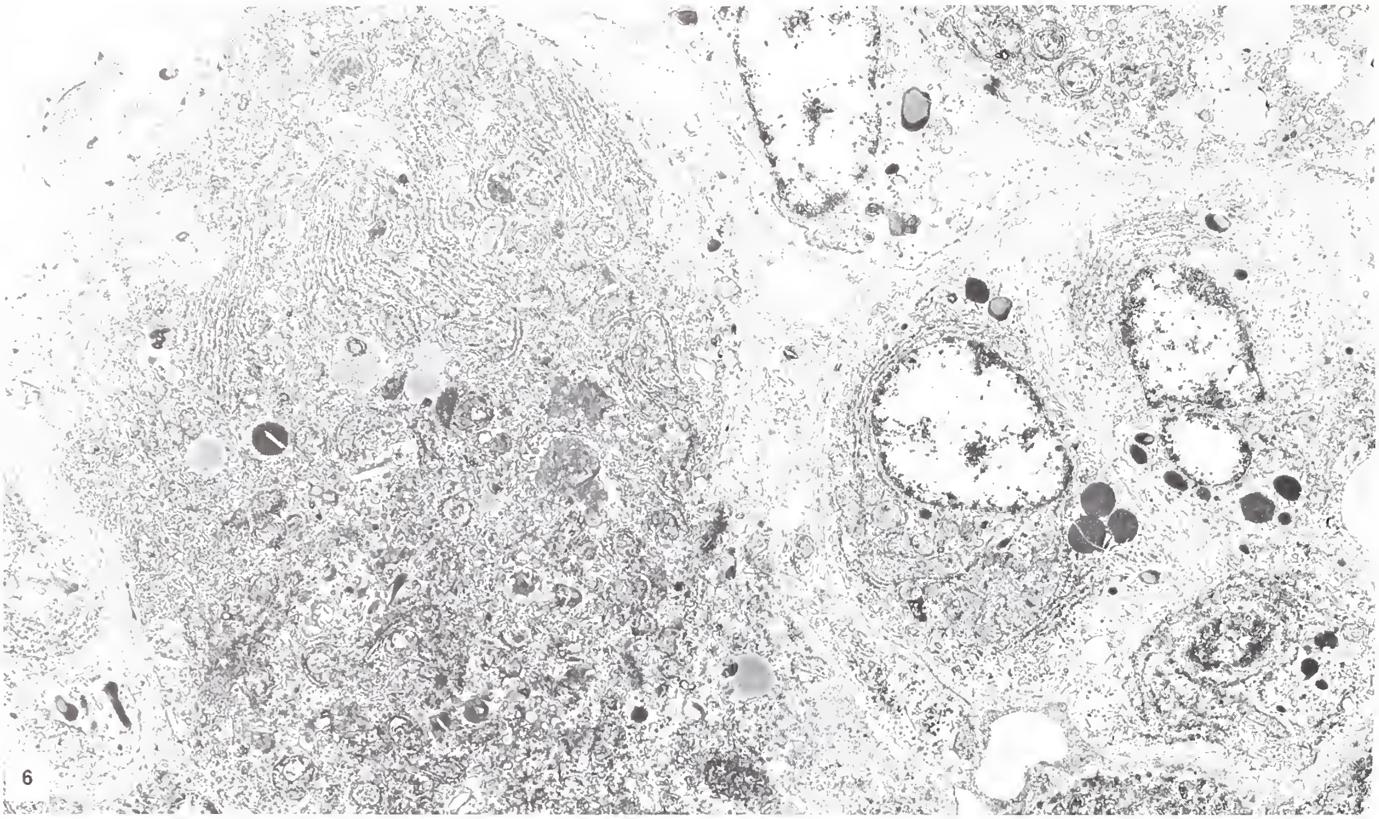

FIGURE 6.—Electron micrograph of a portion of a well-differentiated trabecular hepatoma is shown. Several cells are evident. Large areas of rough endoplasmic reticulum are evident frequently surrounding mitochondria, lipid droplets, and lysosomes. Little glycogen is evident within cytoplasm. × 3,000

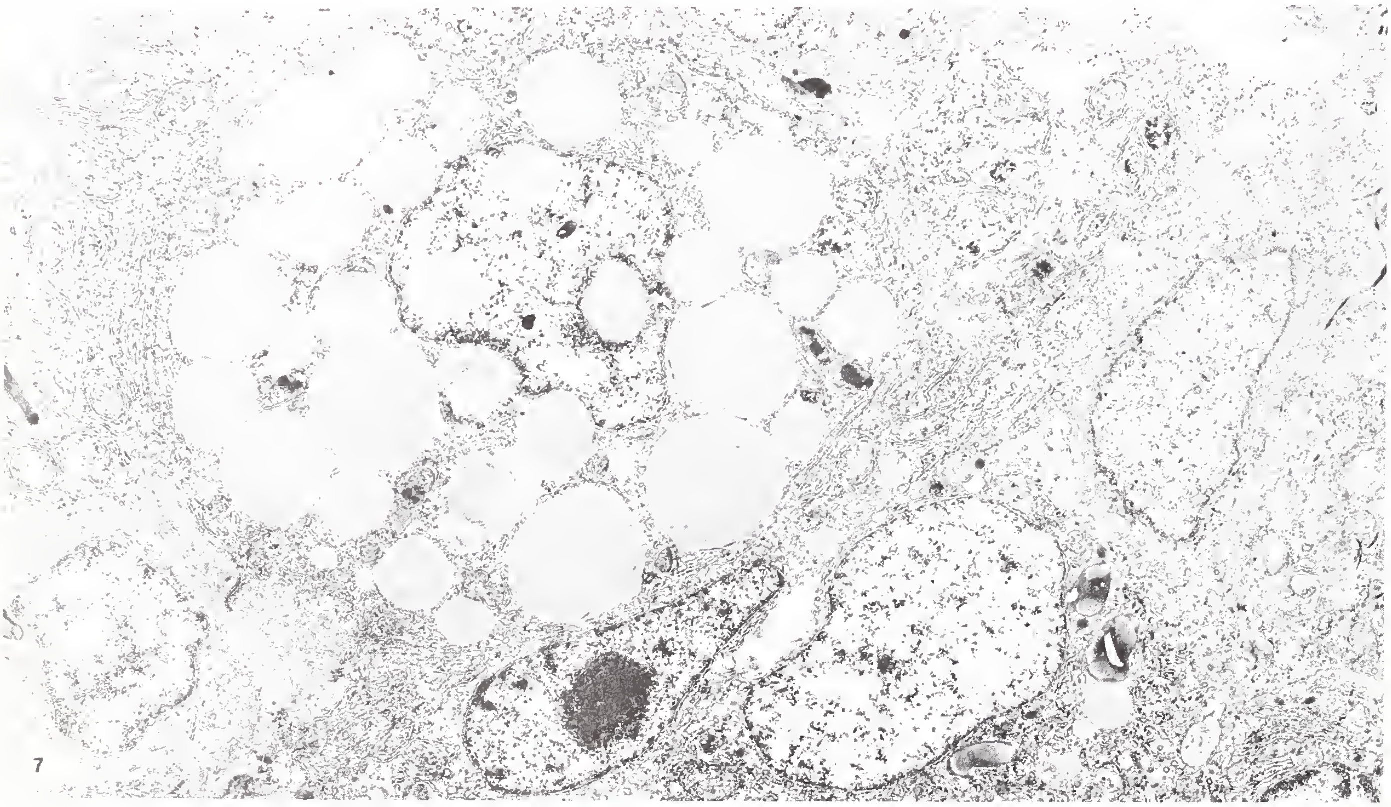

FIGURE 7.—Electron micrograph of a portion of a well-differentiated trabecular hepatoma. *Note* lipid-laden cell. More than 30 lipid droplets of varying size are evident within the cytoplasm and nucleus. × 3,000

DISCUSSION

Wales et al. (*5*) and Hendricks and associates (*6*) reported on the production of hepatic tumors in adult rainbow trout following exposure to a single dose of carcinogen (aflatoxin B_1, *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine, and DENA) during embryogenesis. When the amount of aflatoxin B_1 absorbed by the trout embryos was compared with that consumed by adult fish during a 1-year feeding experiment, the trout embryo was more sensitive to the hepatocarcinogenic action of aflatoxin B_1 than the juvenile trout.

We performed the present investigation to evaluate the potential usefulness of the medaka embryo as a model for chemical carcinogenesis. The medaka offers two potential advantages as a model for embryo carcinogenesis. Firstly, medaka can be induced by photoperiod and temperature adjustments to produce eggs daily (approximately 10–40 eggs/female). These eggs are easily collected and handled. Secondly, the adult medaka has been shown to be an excellent model for hepatocarcinogenesis. Liver tumors were readily inducible in the adult medaka after exposure to several chemical carcinogens including DENA (*3*) and MAM acetate (*4*). Using DENA as the chemical carcinogen in our preliminary study, we demonstrated that medaka embryos may be a valuable model for chemical carcinogenesis. Following continuous DENA treatment during embryogenesis for 10 days, liver tumors were produced in adult fish 3 to 6 months later. The percentage of tumors developed and their time of onset were proportional with the concentration of DENA to which the embryos were exposed.

The light and electron microscopic characteristics of DENA-induced tumors in this study correlated well with those previously described by Ishikawa et al. (*3*). The observed basophilic focal areas of cellular alteration in medaka exposed as embryos to DENA were reminiscent of the hepatic foci in rodent livers induced by chemical carcinogen exposure (*8–11*). The presence of foci in rodent liver has been suggested as a possible preneoplastic lesion. Extensive, further investigation of the fish liver foci would be necessary before the relationship of these lesions to fish hepatocellular neoplasia could be established. Histochemistry and light microscopy studies and morphometric analyses [such as have been performed in the rodent models (*8–12*)] on the teleost focal liver lesions would be needed to provide a better evaluation of their nature.

In summary, we have described our preliminary studies with the medaka embryo as a model for chemical carcinogenesis. The medaka embryo may also be useful in the investigation of potential toxic and teratogenic effects of a particular chemical compound. Further research is needed on the most sensitive stage of embryogenesis to chemical carcinogenesis and the optimal duration of carcinogen exposure.

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Establishment of Fish Hepatocyte Cultures for Use in In Vitro Carcinogenicity Studies[1, 2]

James E. Klaunig[3]

ABSTRACT—Methods were developed for the isolation and primary culture of rainbow trout (*Salmo gairdneri*) and channel catfish (*Ictalurus punctatus*) liver cells. Using a two-step perfusion technique, I isolated an average of 2.75 and 2.87 liver cells/g body weight from the trout and catfish, respectively. Hepatocytes represented 91.4% (trout) and 90.1% (catfish) of the total liver cells isolated. Both catfish and trout hepatocytes in primary culture displayed a linear decrease in survival with increased duration of culture. The DNA synthesis in the hepatocytes during culture showed a similar decrease with increased time in culture. Approximately 2.8% (trout) and 3.5% (catfish) of the hepatocytes exhibited nuclear labeling with [^{3}H]dThd immediately after isolation. The labeling index decreased for hepatocytes from both species to 0% by the tenth day of culture. Activities of cytochrome P-450 and B-5 initially declined rapidly for both trout and catfish hepatocytes after placement in culture; however, these activities leveled off at low but measurable values for the first 8 days of culture. Unscheduled DNA synthesis (UDS) was induced in catfish and trout hepatocytes after exposure to dimethylnitrosamine, aflatoxin B_1, benzo[*a*]pyrene, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Trout hepatocytes displayed a decrease in UDS induction with aflatoxin B_1 with increased age of the cultures. However, UDS induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine remained constant throughout the culture period. — Natl Cancer Inst Monogr 65: 163–173, 1984.

The liver is known to be the major site of the tumorigenesis in teleosts following their exposure to most chemical carcinogens (*1–3*). Various carcinogenic compounds including DMNA (*4, 5*), diethylnitrosamine (*6, 7*), AFB_1 (*4, 8, 9*), *N*-2-fluorenylacetamide (*4*), and 7,12-dimethylbenz[*a*]anthracene (*10*) have induced hepatic neoplasms in fish. These findings are not surprising because most of the marine and freshwater fish studied possess measurable and inducible mixed function oxidase systems (*10–13*), which are necessary for the activation of most carcinogens.

During the past decade, methodology has been developed for the isolation and primary culture of hepatocytes from adult rats (*14, 15*) and adult mice (*16, 17*). These cells have proved valuable for use in the study of the mechanisms of carcinogenicity and in short-term in vitro mutagenesis assays. Metabolism (*18*) and macromolecular binding of carcinogens (*19*) and carcinogen-induced DNA repair (*20*) have been investigated in vitro with the use of cultured rodent liver cells. The advantages of such use of freshly isolated or cultured hepatocytes, or both, from vertebrate livers have been reviewed (*21*). The morphological and functional components of short-term cultured rodent hepatocytes are similar to those found in vivo. Carcinogen metabolism by freshly isolated rat hepatocytes more closely resembled that of the in vivo liver than did subcellular liver homogenate preparations (*21*). Therefore, liver cells in short-term culture may be a useful system with which one can study the mechanisms of liver carcinogenesis.

Recently, several investigators applied the methodology of rodent liver cell isolation to teleosts and studied the functional behavior of freshly isolated rainbow trout liver cells. Walton and Cowey (*22*) investigated the effects of several agents, including glucagon and quinolinate, on gluconeogenesis in isolated trout liver cells. Porthe-Nibelle and Lahlou (*23*) examined the uptake of cortisol and dexamethasone in isolated rainbow trout liver cells. Meanwhile, Bailey et al. (*24*) used isolated trout liver cells to study the mechanisms of AFB_1 adduct formation in the fish.

We initiated the present study to 1) establish methodology for the isolation and culture of viable hepatocytes from trout and channel catfish, 2) characterize the function of these cells in culture, and 3) investigate the ability of these cells to repair DNA following exposure to direct- and indirect-acting chemical carcinogens.

MATERIALS AND METHODS

Animals.—Male rainbow trout (*Salmo gairdneri*) weighing 150–200 g each and male channel catfish (*Ictalurus punctatus*), 100–150 g each, were used. The rainbow trout were supplied by a local commercial trout farm (Castalia

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ABBREVIATIONS: DMNA = dimethylnitrosamine; AFB_1 = aflatoxin B_1; L-15 = Leibowitz's L-15 medium; HIFBS = heat-inactivated fetal bovine serum; HRLM = high-resolution light microscopy; TEM = transmission electron microscopy; UDS = unscheduled DNA synthesis; BP = benzo[*a*]pyrene; MNNG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DMSO = dimethyl sulfoxide.

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Farms, Castalia, Ohio); catfish were purchased from a local supplier. Both species were held separately in 100-gallon Living Stream Aquaria (Frigid Units, Inc., Toledo, Ohio). Water temperature was maintained at 15° C for trout and 22° C for catfish. Fish were fed a commercial trout chow twice daily; all were maintained in the laboratory for at least 2 weeks before the cell isolation experiments.

Liver cell isolation.—Trout and channel catfish were anesthetized with tricaine methanesulfonate in dechlorinated water, placed on a surgical board (ventral surface up), and swabbed on the ventral surface with 70% ethanol (fig. 1A). A V-shaped incision was made above the heart, and the underlying tissue was removed, exposing the heart and ventral aorta (fig. 1B). A second incision along the linea alba was made from the pectoral girdle to the urogenital pore. We exposed the viscera by continuing this incision laterally on each end, thus allowing the ventral body wall to be reflected to either side. A branch of the hepatic portal vein was located and a loosely tied ligature was placed around it approximately 20 mm posterior to its entrance into the liver. We inserted a plastic cannula in the portal vein just posterior to the ligature (fig. 1C). The ligature was tightened around the cannula and perfusion with Hanks' calcium- and magnesium-free salt solution containing 0.5 m*M* ethyleneglycol-bis(β-aminoethyl ether) *N*-*N'*-tetraacetic acid and 0.05 *M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.3) was begun. After the liver swelled, a small cut was made in the ventral aorta 5 mm anterior to the bulbus arteriosus. This incision relieved the perfusion pressure build-up caused by the Hanks' perfusion and allowed an outflow for the perfusate; the liver returned to normal size as a result of this incision. With a pair of blunt-tipped forceps (fig. 1D), the bulbus arteriosus was grasped posterior to the incision, which resulted in the occlusion of the ventral aorta and the subsequent reswelling of the liver. The forceps were then released for drainage of the perfusate. Following liver shrinkage, the ventral aorta was again occluded. This intermittent clasping of the ventral aorta was continued at 15-second intervals for the duration of the perfusion. After 4–5 minutes of perfusion with Hanks' solution, the perfusate was changed to a collagenase solution (100 U of collagenase/ml of L-15 (Sigma type I, 165 U/mg; Sigma Chemical Co., St. Louis, Mo.). Perfusion with the collagenase-containing medium continued for 10–12 minutes. A perfusion rate of 10 ml/minute was maintained for both perfusates. The 2 solutions were incubated at 30° C during the perfusion, after which the liver was carefully excised and placed in a 100×20-mm sterile plastic petri dish containing 10 ml of collagenase perfusate (also at 30° C). We carefully removed the gallbladder to prevent bile leakage, and the liver cells were dissociated from the liver stroma by combing with a stainless steel dog comb (fig. 1E). Following the combing, the remaining fibrous plug was removed, and the liver cell suspension was pipetted ten times with a wide-bore 10-ml pipette for further dissociation of the liver cells (fig. 1F). The cells were first passed through nylon mesh (Nitrex HC3-253; TETCO, Inc., Elmsford, N.Y.) into a 50-ml sterile centrifuge tube and then pelleted at 100×*g* for 5 minutes at 15° C. The supernatant was discarded and the pellet was resuspended in 40 ml of complete medium (L-15 with 10% HIFBS and 25 μg gentamycin/ml). An aliquot of the cell suspension was mixed 1:5 with trypan blue (0.4% in saline), incubated for 5 minutes at 22° C and counted on a hemocytometer. The total number of cells and percentage viable cells (trypan blue exclusion) were calculated for each isolation.

Isolated cell morphology.—Isolated trout and channel catfish liver cells were morphologically evaluated by phase contrast light microscopy, HRLM, and TEM. For phase contrast microscopy, cells were plated onto a 60-mm plastic petri dish and viewed under a Leitz Diavert Inverted phase contrast microscope. For HRLM and TEM, cells were fixed in 3% glutaraldehyde buffered with 0.2 *M* cacodylate (pH 7.4) for 4 hours, postfixed in 1% osmium tetroxide for 30 minutes, dehydrated in increasing concentrations of ethanol followed by propylene oxide, and embedded in Epon 812. Representative sections, 0.5–1.0 μ thick, were cut for HRLM on an ultramicrotome and stained with toluidine blue. Ultrathin sections for TEM were cut on an ultramicrotome, mounted on 300-mesh copper grids, stained with uranyl acetate and lead citrate, observed, and photographed on a Ziess 9S electron microscope.

Cell survival.—As previously noted (*25*), trout and channel catfish hepatocytes did not attach securely to plastic culture dishes. Our attempts to enhance the attachment of these cells by varying the serum concentration of the medium, the duration of attachment, or the types of sera or substrate used were generally ineffective. However, trout and catfish hepatocytes remained viable in a suspension-type culture system. Therefore, cell survival of trout and catfish cells in suspension culture was determined. Freshly isolated trout and catfish liver cells were plated onto 60-mm culture dishes at a concentration of 2.0×10⁶ viable cells/dish. Then 5 ml L-15 containing 10% FBS was added to each dish. Cells were incubated at 25° C in a humidified 100% air incubator. For a change in medium, liver cells were removed from each culture dish and centrifuged at 100×*g* for 5 minutes. The supernatant was discarded, and the resulting pellet was resuspended in 5 ml of fresh medium. Medium was changed after 2 days and every 3 days thereafter. To determine the surviving cell number, we counted the cells from 5 dishes at 4 hours, 1 day, and 4, 6, 8, and 14 days of culture. Cells were pipetted from each culture dish and placed in a 15-ml centrifuge tube. Each dish was washed three times with 3 ml of fresh medium for removal of any remaining cells. These washings were added to the pipetted combined cells and centrifuged at 100×*g* for 5 minutes at 10° C. The supernatant was removed, the cells were resuspended in fresh serum-free L-15, and 3 aliquots of the resuspended cells were counted on a hemocytometer with trypan blue exclusion as the parameter for viability. The average number of viable and nonviable cells was determined for the 3 aliquots. Five dishes were counted at each sampling period for each experiment. A total of 10 experiments (or 10 separate liver cell isolations) were performed for both the trout and channel catfish.

DNA synthesis.—The percentage of trout and channel catfish hepatocytes undergoing DNA synthesis during the

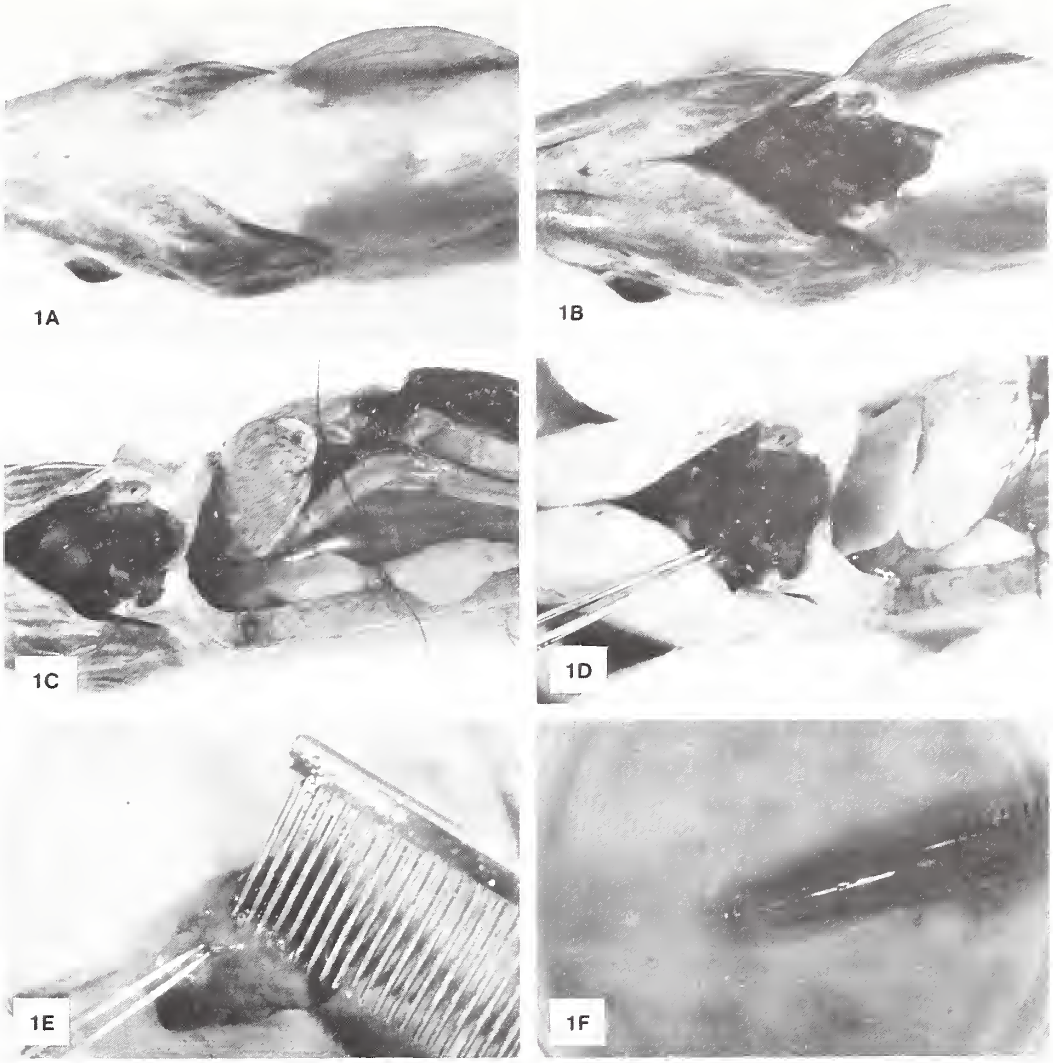

FIGURE 1.—Photographs of the rainbow trout liver cell isolation procedure. A) Anesthetized trout was placed on a surgical board with the ventral surface up. B) A V-shaped incision was made above the heart. C) Viscera was exposed and a branch of the hepatic portal vein was cannulated. D) Bulbus anteriosus was intermittently occluded with blunt forceps. E) Following perfusion with collagenase, the liver was removed, and liver cells were liberated by combing. F) After combing, the cell suspension was repeatedly pipetted for further dissociation of the clumps of liver cells.

first 14 days of culture was also evaluated. Trout and catfish liver cells (2.0×10^6 viable cells) were plated onto 60-mm plastic petri dishes, and 5 ml of L-15 with 10% HIFBS and 25 μg gentamycin/ml were added to each dish. The dishes were incubated in a humidified 100% air incubator at 25° C. The medium was changed after 2 and 4 days and every 3 days thereafter. Triplicate dishes were treated with [^{3}H]dThd (10 μCi/ml; 50 Ci/mmol) at zero time, 1 day, and 2, 4, 6, 8, and 14 days in culture. After a 4-hour exposure to [^{3}H]dThd, the cells were transferred to 16×150-mm sterile glass test tubes and centrifuged at $100\times g$ for 5 minutes. The resulting pellet was resuspended in 5 ml of complete L-15. This process was repeated three times so that the unbound radiolabeled dThd was removed. The final pellet was resuspended in 2 ml of serum-free L-15, and the cells from each tube were pelleted on 3 clean glass slides by centrifugation. The cells were fixed by brief immersion in 1:3 acetic acid: ethanol and allowed to air dry. The slides were dipped in NTB-2 autoradiographic emulsion (Eastman Kodak Co., Rochester, N.Y.) and stored in dry black Bakelite slide boxes at 4° C for 2 weeks. The autoradiographs were developed with D-19 (Eastman Kodak Co.) and stained with hematoxylin and eosin. One thousand cells/slide were counted at random, and the number of cells undergoing DNA synthesis (as evidenced by blackened nuclei) were scored. Results were based on liver cell isolations of 5 trout and 5 catfish with a total of 3 slides counted per isolation for each sampling period.

Cell morphology.—Trout and catfish cells were morphologically evaluated at 1 day, and 2, 4, 6, 8, and 14 days of culture by phase contrast light microscopy, HRLM, and TEM. Cells examined by HRLM and TEM were fixed with 3% glutaraldehyde in 0.2 *M* cacodylate buffer (ph 7.4) for 4 hours, postfixed in 1% osmium tetroxide, dehydrated in increasing concentrations of ethanol, and embedded in Epon 812. Representative sections, 0.5–1.0 μ thick, were cut for HRLM and stained with toluidine blue. Ultrathin sections for electron microscopy were cut on an ultramicrotome, mounted on 300-mesh copper grids, stained with uranyl acetate and lead citrate, observed, and photographed on a Zeiss 9S electron microscope.

Cytochrome P-450 and B-5 determinations.—Trout and channel catfish hepatocytes were assayed for these cytochrome concentrations during primary culture. Isolated fish liver cells were plated at a concentration of 2×10^6 viable cells/60-mm petri dish, and then 5 ml of L-15 containing 10% FBS and 25 μg gentamycin/ml were added to each dish. Cells were grown in suspension culture at 25° C with medium changes after 2, 4, and 7 days. Immediately after plating onto the culture dish (zero time), at 1 day, and 2, 4, 6, and 8 days of culture, trout and channel catfish hepatocytes were sampled for cytochrome P-450 and B-5 concentrations. Five dishes of cells from each species were pooled into a test tube to form 1 data point. A total of 3 data points were determined for each species at each of the 6 sampling periods. Sampled cells were pelleted by centrifugation ($100\times g$ for 5 min). The supernatant was removed, and the cells were resuspended in phosphate-buffered saline. Cells were washed three times (5 min/each wash) in 10 ml of phosphate-buffered saline. Following the washing, cells were homogenized at 4° C by 5 strokes of an Elvehjem-Potter tissue homogenizer. The homogenates of each tube were quickly frozen in liquid nitrogen and stored at −80° C until P-450 and B-5 analyses were performed.

Cytochrome P-450 and B-5 assays were performed on undiluted, thawed, fish liver cell homogenate according to the methods of Omura and Sato (*26*); DNA was assayed on an aliquot of each homogenate by the method of Bonney et al. (*27*).

Unscheduled DNA synthesis determination.—We measure UDS in trout and catfish liver cell cultures following exposure to either DMNA, AFB_1, BP, or MNNG. Freshly isolated trout and channel catfish hepatocytes were plated (2.0×10^6 viable cells) onto 60-mm plastic petri dishes. After 2 hours of culture, DMNA, AFB_1, BP, or MNNG dissolved in DMSO was added to the culture dishes at 1 of 3 concentrations (ranging from 10^{-2} *M* to 10^{-6} *M*) based on previous toxicity studies. Three dishes of cells were treated with each concentration of carcinogen. Controls included the solvent vehicle (DMSO) and no treatment. The amount of carcinogen and DMSO added to each dish at no time represented more than 0.1% of the total medium volume. One-half hour before treatment with the carcinogens, [^{3}H]dThd (10 μCi/ml, 50 Ci/mmol) was added to each dish. The two control groups were also treated with [^{3}H]dThd. Hepatocyte cultures, treated with carcinogen and [^{3}H]dThd for 24 hours, were then placed in a 15-ml tube and centrifuged at $100\times g$ for 5 minutes (10° C). The supernatant was removed, and the resulting cell pellet resuspended in fresh unlabeled medium. After 20 minutes, the cell suspension was recentrifuged ($100\times g$ for 5 min), and the pellet was resuspended in fresh medium. This washing procedure was repeated three times (20 min each). After the final washing, the pelleted cells were resuspended in 2 ml of serum-free L-15. Aliquots (0.5 ml each) of this suspension were placed on clean glass microscope slides with a clinical cytocentrifuge; 3 slides were prepared from each tube. Slides were dipped in a solution of acetic acid: ethanol (1:3) for cell fixation. We allowed the cells to air dry and then processed them for autoradiography by dipping the slides in NTB-2 liquid emulsion (Eastman Kodak Co.). Dipped slides were stored in black Bakelite boxes for 2 weeks at 4° C. Autoradiograms were developed in D-19 developer (Eastman Kodak Co.), stained with hematoxylin and eosin and viewed under a light microscope at × 1,000 (under oil immersion). We quantified UDS by counting the number of nuclear grains in 100 random consecutive cells/slide. In binucleated cells, each nucleus was counted separately. The number of net nuclear grains was determined for each cell; net grains were the number of nuclear grains/cell minus the average number of grains counted in 3 nuclear-sized areas from the adjacent cytoplasm. Replicative DNA synthesis was evidenced by a complete blackening of the nucleus with grains too numerous to count.

In previous studies in our laboratory, UDS induction in mouse liver cells by chemical procarcinogens was decreased with increased age of the cell cultures. In our present study, this phenomenon was similarly investigated with trout and catfish liver cells. The MNNG, BP, and AFB_1 were tested for UDS induction in trout and catfish liver cells after 4 hours, 2 and 4 days of culture. At each sampling time, cells were treated concomitantly with [^{3}H]dThd and the test carcinogen for 24 hours. After exposure, the cells were washed and processed for autoradiographic analysis as detailed above.

RESULTS

Cell Isolation

The results of 37 trout and 43 channel catfish liver cell isolations are shown in table 1. Means of 2.75 and 2.87 total liver cells/g body wt[5] with an average viability of 94.4 and 96.7% were obtained for the trout and catfish, respectively. The percentage of trout and channel catfish hepatocytes in the isolates was determined by a count at random of the number of hepatocytes and nonhepatocytes in HRLM preparations of 5 liver cell isolates for each species of fish. A total of 1,000 cells were counted from each of the liver isolations. Hepatocytes represented 91.4 and 90.1% of the

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[5] The use of cells/gram body weight is a more accurate method for one to estimate cell yield from animal to animal. Wet liver weights are not accurate because a variable amount of perfusate remains in the swollen liver after perfusion. The percentage of body weight that is liver remains fairly constant in adult animals (of the same sex). The body weight is easily determined and because it has a repeatable relationship to the liver weight, it is a good standard for expression of cell yield.

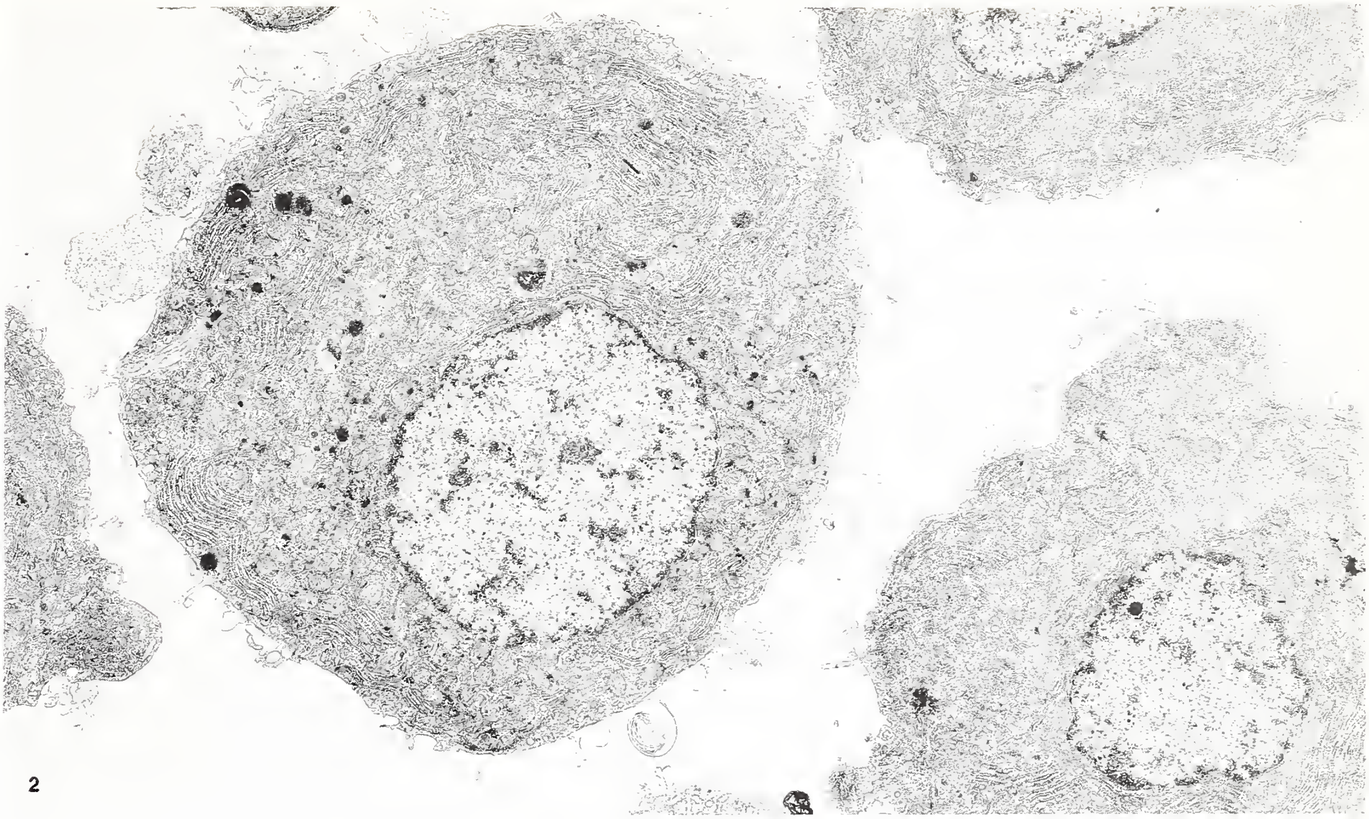

FIGURE 2.—Electron micrograph of a freshly isolated trout liver cell pellet. Cells are individual, spherical, and morphologically similar to in vivo hepatocytes. Cytoplasm contained numerous profiles of rough endoplasmic reticulum and oval mitochondria. A single nucleus was usually centrally located within the isolated cells. × 3,000

total cells isolated for the trout and channel catfish, respectively. The remainder of the isolate consisted of exocrine pancreatic cells (6.3% for trout and 7.4% for channel catfish) and other cells (2.3% for trout and 2.5% for catfish). Figure 2 is an electron micrograph of trout hepatocytes immediately following isolation.

The viability of the isolated trout and catfish liver cells during storage at 4° C was also examined (table 2). Viability of liver cells from both fish species decrease with increased time in storage. An approximate 8% loss of viability was observed in the liver cells after 1 day of storage, whereas a loss of 13–14% in viability was demonstrated after 2 days.

Cell Survival

Figure 3 shows the percentage of surviving trout and catfish liver cells during the first 14 days of primary culture; liver cells from both species displayed a gradual decrease in viability over time. The viability ranged from 96.3 for trout and 92.7% for catfish after 1 day of culture to 20.6 and 15.2% after 14 days, respectively. Phase contrast microscopy of catfish liver cells during the primary culture period is depicted in figure 4. Catfish liver cells immediately after placement in culture (zero time) were individual and rounded (fig. 4A). Liver cells after 2 days of culture formed small groups of 2–20 cells in size (fig. 4B). Individual cells were also present. After 4 days (fig. 4C), these groups became larger and contained up to 100 cells each. Groups of catfish liver cells after 8 days of culture assume a more oval morphology frequently surrounded by a dense membrane-like structure (fig. 4D). Trout liver cells in culture displayed morphological characteristics identical to those observed for the catfish. The viability of the cells within the groups (determined by trypan blue exclusion) showed a variable pattern of distribution. Cells on the exterior of the

TABLE 1.—*Fish liver cell isolation*[a]

Fish	No. of isolations	Total No. of isolated cells/g body wt ($\times 10^6$)	Mean viability, %	Total No. of viable hepatocytes/g body wt ($\times 10^6$)
Trout	37	2.75±0.47	94.4	2.31±0.21
Catfish	43	2.87±0.59	96.7	2.50±0.30

[a] Values represent the mean ± SD.

TABLE 2.—*Viability of isolated fish liver cells in storage*[a]

Time in storage, hr	Mean percent viabilities in 10 cell isolations	
	Trout	Catfish
0	95.2	96.1
4	93.1	96.0
8	92.1	94.2
12	88.0	89.5
24	87.2	87.5
48	82.3	82.0

[a] Fish liver cells were placed in 100 ml of complete L-15 (2.0×10^6 viable cells/5 ml medium) and stored at 4° C.

groups as well as those internally within the groups stained with trypan blue. However, a greater percentage (over 70%) of the trypan blue-stained cells were found internally.

Channel catfish cells during primary culture were also examined by TEM. After 1 day of culture, most of the cells were individual; a single, centrally located nucleus was evident in most cells. The cytoplasm was predominantly filled with oval-shaped mitochondria and parallel strands of rough endoplasmic reticulum. Frequently, portions of the cytoplasm near the plasma membrane contained clear spaces. Lipid droplets and lysosomes were occasionally seen within the liver cells.

After 4 days of culture, most catfish liver cells were in groups that displayed variable morphologies (fig. 5). Nuclei were frequently elongated and contained a serrated outer membrane. The cytoplasm contained numerous mitochondria, profiles of rough endoplasmic reticulum, and large autophagic or heterophagic, or both, vacuoles. These vacuoles were frequently as large as the nucleus. Junctional complexes were evident between cells. Occasional bundles of 100-angstrom, intermediate-type microfilaments were present within the liver cell cytoplasm. Large lipid droplets were also observed in the cytoplasm. After 8 days of culture, the cells within the groups displayed a morphology similar to that seen earlier (after 4 days). The most dominant cytoplasmic feature was the presence of large phagosomes within the cytoplasm of most cells (fig. 6). These vacuoles frequently occupied one-half to one-third of the cell. Junctional complexes between neighboring cells were evident. Necrotic cells were frequently found in the interior of the groups.

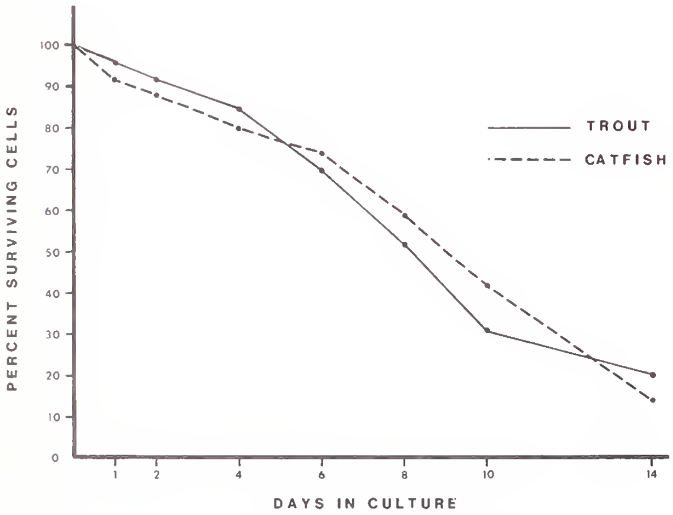

FIGURE 3.—Graph displays the pattern of catfish and trout liver cell survival during the first 2 wk of primary culture. Viability of trout and catfish liver cells decreased almost linearly during the culture period.

DNA Synthesis

When the percentage of trout and catfish liver cells in DNA synthesis during the first 14 days of primary culture was investigated (fig. 7), we found that immediately after isolation, the cells displayed a labeling index of 2.8 and 3.5% for the trout and catfish, respectively. The labeling of trout liver cells dropped to 1% by 1 day of culture and gradually decreased to 0% by day 10. Catfish liver cells showed a similar pattern, displaying a labeling index of 0.7% after 1 day, with a progressive decrease to 0% by 8 days. No labeling was observed in either trout or catfish liver cells after 14 days of culture.

Activities of Cytochromes P-450 and B-5

Trout liver cells assayed immediately after isolation exhibited average activities of 1.05 and 0.82 pmol/mg DNA for cytochromes B-5 and P-450, respectively, whereas these activities in catfish liver cells amounted to 1.14 and 0.99 pmol/mg DNA B-5 and P-450 (fig. 8). A rapid decrease was noted in B-5 and P-450 activities in the liver cells from both species after the first day in primary culture. Both enzymes decreased proportionately during this period in the catfish and trout liver cells, resulting in activities of less than 30% of those present in the initial isolate. A more gradual decrease in activity was observed for both enzymes from day 1 to 4 of culture after which the cytochrome activity leveled off at values approximately 15 to 20% (for B-5) and 6 to 10% (for P-450) of those measured in the freshly isolated cells.

Unscheduled DNA Synthesis

As evidenced by UDS induction, DNA repair was examined in trout and catfish liver cells following exposure to DMNA, AFB_1, BP, and MNNG. Each carcinogen was tested at 3 concentrations. Positive UDS was induced by all 4 compounds in liver cells from both fish species (table 3). Trout liver cells demonstrated a higher mean number of grains per nucleus than did those of the catfish after exposure to DMNA and AFB_1. Catfish liver cells, meanwhile, displayed a higher net nuclear labeling than did those of the trout after treatment with BP. The number of net nuclear grains induced by MNNG were not significantly different in the 2 species. Positive UDS induction displayed a dose response for all the carcinogens studied.

An evaluation of the influence of age of the cultures on UDS induction by MNNG and AFB_1 in trout liver cells (table 4) revealed that after 4 hours and 2 or 4 days, cells in culture exposed to either carcinogen for 24 hours showed a

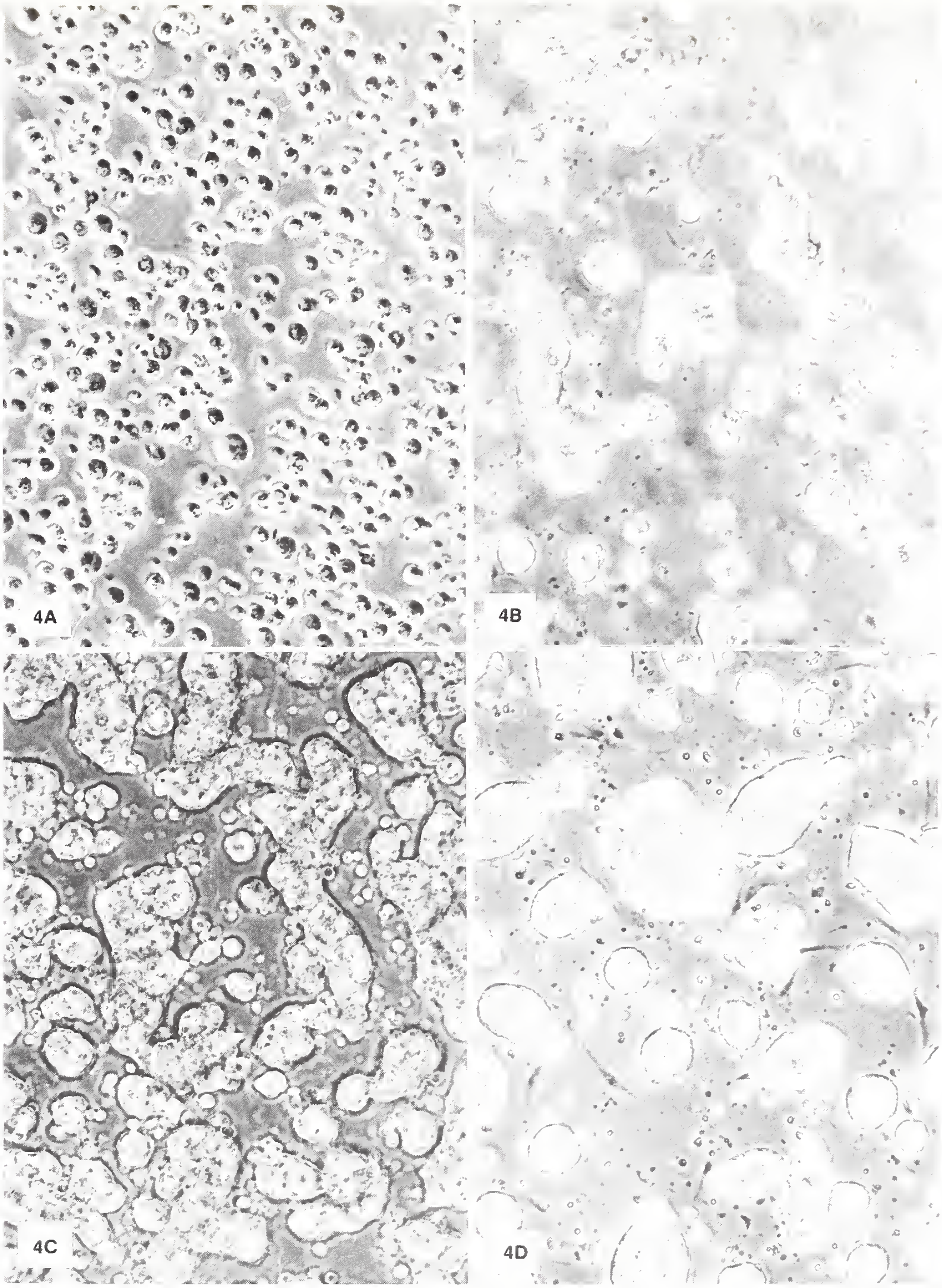

FIGURE 4.—Phase contrast photomicrograph of catfish liver cells during suspension culture. × 100. A) Catfish liver cells after placement in culture were individual and spherical. B) After 2 days of culture, most cells were in small, floating groups. C) Groups of liver cells became larger after 4 days of culture. D) Catfish liver cells after 8 days of culture were present in large groups.

significant increase in net nuclear grains at all 3 concentrations tested. Trout liver cells after 2 and 4 days of culture displayed a similar dose-related response to MNNG induction of UDS. No significant difference was noted in the number of net nuclear grains induced by MNNG exposure after 4 hours or 2 or 4 days of culture. The effect of AFB_1 on UDS induction in the trout liver cells decreased significantly with time in culture. After 4 hours of culture, all 3 dose levels of AFB_1 tested induced a significant increase in the number of net nuclear grains. After 2 days of

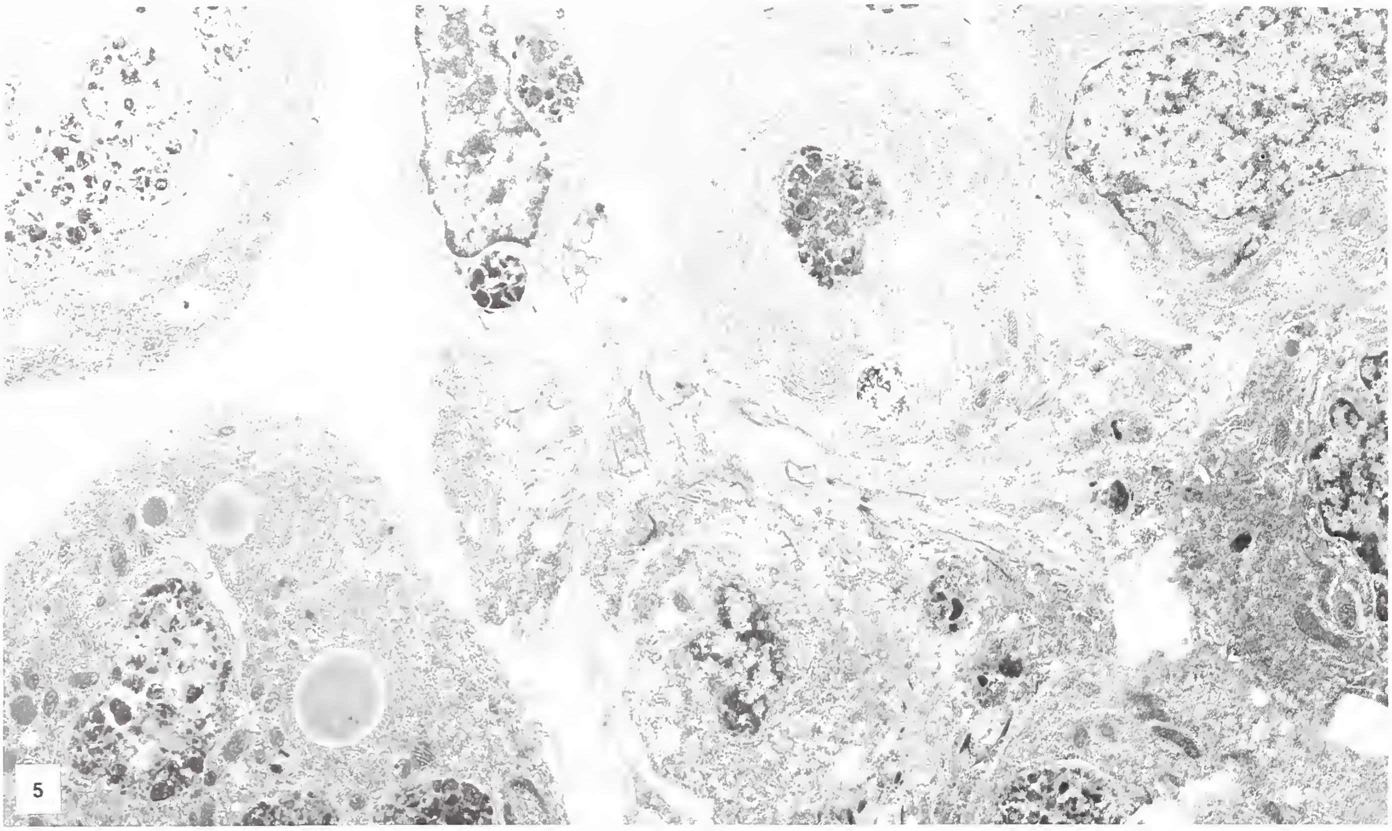

FIGURE 5.— Electron micrograph of channel catfish liver cells after 4 days of culture. Cells within the group formed junctional complexes with each other. Liver cell cytoplasm contained multiple profiles of rough endoplasmic reticulum, oval mitochondria, large autophagic vacuoles, and occasional lipid droplets. Nuclei were frequently elongated. × 3,000

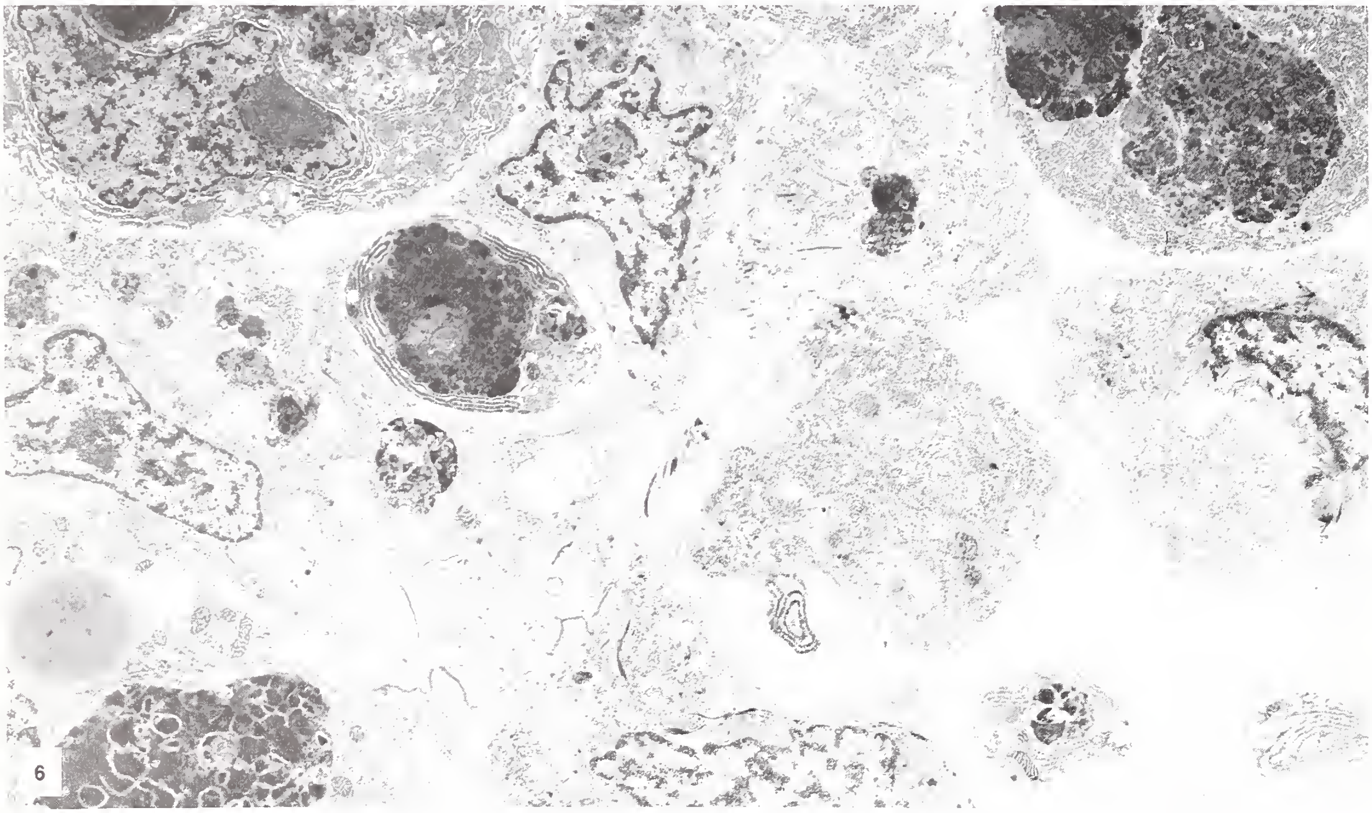

FIGURE 6.—Electron micrograph of channel catfish liver cells after 8 days of culture. Cells in groups contained large autophagic vacuoles. Nuclei were frequently indented with cytoplasmic invaginations. × 3,000

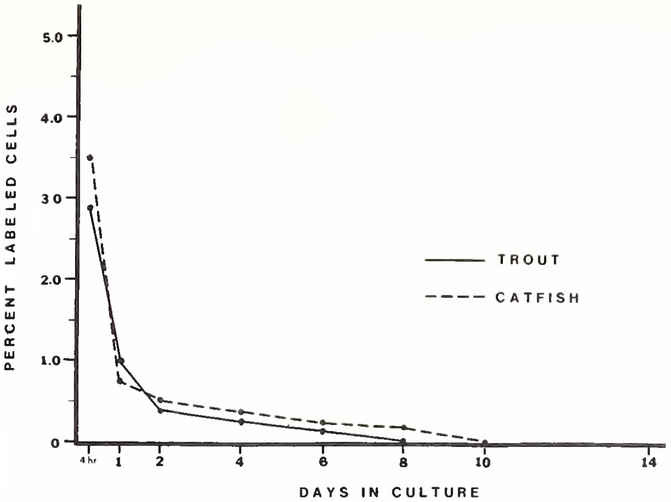

FIGURE 7.—DNA synthesis in trout and catfish liver cells during the first 14 days of culture. The percent of labeled cells (as evidenced by the blackened nuclei) was determined in 3,000 randomly counted cells from each isolation.

TABLE 3.—*UDS induction in freshly isolated trout and catfish liver cells*

Compound	No. of net grains/nucleus[a]		
	Concentration, *M*	Trout	Catfish
Control	—	0.32±0.09	0.21±0.08
DMNA	1×10^{-4}	4.33±0.53	0.99±0.10
	1×10^{-3}	5.51±1.30	1.90±0.89
	1×10^{-2}	6.42±1.35	3.40±1.02
AFB$_1$	1×10^{-5}	6.01±0.75	1.21±0.63
	1×10^{-4}	7.79±0.83	1.60±0.49
	1×10^{-3}	9.20±0.87	2.04±0.44
BP	1×10^{-5}	2.20±0.80	2.91±0.81
	1×10^{-4}	2.85±0.87	5.37±2.01
	1×10^{-3}	4.01±1.20	6.32±2.41
MNNG	1×10^{-6}	2.31±0.84	2.67±0.86
	1×10^{-5}	3.24±1.08	3.05±1.04
	1×10^{-4}	5.50±1.31	4.45±1.05

[a] No. of net grains was determined by the No. of grains/nucleus minus the mean of 3 adjacent similar sized cytoplasmic areas; 100 cells were counted/slide and 3 slides were counted/concentration (24 hr exposure). All carcinogen exposure values were significantly increased over the control ($P<0.001$).

culture, only 1×10^{-4} and 1×10^{-3} *M* AFB$_1$ concentrations elicited positive UDS. An AFB$_1$ concentration of 1×10^{-5} *M* showed no significant increase in UDS over control values. The amount of UDS elicited by 1×10^{-4} and 1×10^{-3} *M* AFB$_1$-treated liver cells was significantly decreased from that observed in cells after 4 hours of culture. Trout liver cells in culture for 4 days displayed no UDS induction after 1×10^{-5} and 1×10^{-4} *M* AFB$_1$ treatment. However, an AFB$_1$ concentration of 1×10^{-3} *M* induced UDS in the trout liver cells after 4 days. The number of net nuclear grains elicited by the 1×10^{-3} *M* AFB$_1$ concentration was significantly decreased from that observed for the same AFB$_1$ concentration after 4 hours and 2 days of culture.

DISCUSSION

This study demonstrated that rainbow trout and channel catfish liver cells could be isolated and maintained for up to 2 weeks in primary culture. The development of an isolated liver cell model in fish offers several advantages: 1) Studies on the cellular mechanisms of carcinogenesis in teleosts

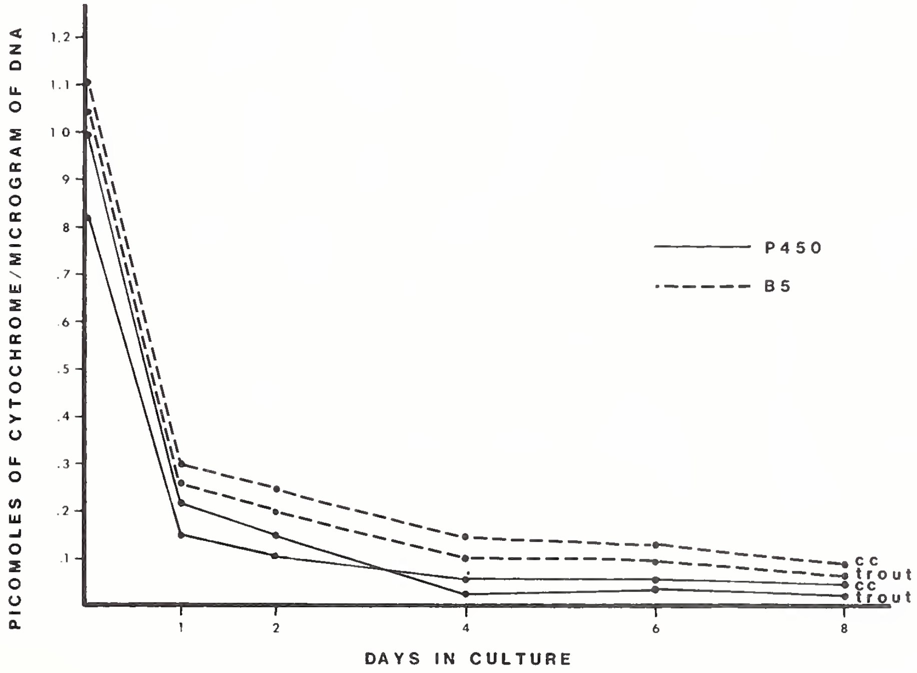

FIGURE 8.—Trout and channel catfish (cc) liver cell cytochrome P-450 and B-5 activities during primary culture.

TABLE 4.—*Effect of age of trout hepatocyte cultures on UDS induction*

Compound	Concentration, *M*	Time in culture at start of exposure[a]		
		4 hr	2 days	4 days
MNNG	1×10^{-6}	2.40 ± 0.89	2.11 ± 0.71	2.50 ± 1.02
	1×10^{-5}	3.51 ± 0.95	3.44 ± 0.98	3.25 ± 1.11
	1×10^{-4}	5.43 ± 1.32	5.68 ± 1.41	5.75 ± 1.03
AFB	1×10^{5}	6.10 ± 1.02	0.20 ± 0.41[b]	0.34 ± 0.10[b]
	1×10^{4}	8.24 ± 1.57	2.61 ± 0.84	0.64 ± 0.43[b]
	1×10^{3}	9.40 ± 2.71	3.89 ± 1.63	2.78 ± 1.21
Control	0	0.37 ± 0.10	0.40 ± 0.14	0.34 ± 0.19

[a] Values represent the mean No. of net nuclear grains determined by the No. of grains/nucleus minus the mean of 3 adjacent similar sized cytoplasmic areas; 100 cells were counted at random/slide and 3 slides were counted/concentration (24 hr exposure). All carcinogen exposure values were significantly increased over the control ($P<0.001$) except as noted by *b*.

[b] $P>0.05$.

can be performed. 2) Intraspecies and interspecies evaluations of a carcinogen's metabolism, activation, binding and damage to DNA, and DNA repair can be studied. 3) Isolated liver cell preparations from teleost liver can be used as a metabolism source in short-term in vitro carcinogenesis and mutagenesis assay systems in lieu of subcellular preparations. Intact liver cells have longer viability and mimic the in vivo uptake and metabolism of chemical carcinogens better than do subcellular liver preparations (*21*).

The methodology we used in the present study for the isolation of trout and catfish liver cells generally followed that reported for the rat (*14*) and mouse (*16*). Isolated trout and catfish liver cells displayed a high viability (averaging 95%) with greater than 90% of the isolated cells being hepatocytes. The isolated cells were for the most part single and intact and maintained a high viability (over 80%) when stored for up to 2 days at 4° C. This capability may allow for the use of a single isolation of cells for several experiments over a 2-day period. Similar isolated liver cell preparations have been used for the study of gluconeogenesis (*22*) and corticosteroid interaction (*23*) in the rainbow trout. More recently, Bailey et al. (*24*) have characterized the formation of AFB$_1$ DNA adducts in isolated trout hepatocytes. Isolated liver cell preparations are valuable tools for the in vitro investigation of the acute effects of carcinogens and mutagens.

We also demonstrated the ability to maintain catfish and trout hepatocytes in primary culture. Unlike their rodent counterparts, both catfish and trout liver cells never exhibited a firm attachment with the culture substrate. Further investigation of this observation is currently ongoing in our laboratory. The teleost cells in suspension culture mimicked the survival performance of rodent hepatocytes attached to plastic (*15, 17*). As with their rodent counterparts, the rainbow trout and channel catfish hepatocytes did not appear to grow or divide in culture. Morphologically, the single cells began to form groups soon after placement in culture that became grossly visible in the petri dish after several days. When viewed under the electron microscope, the viable hepatocytes within a group formed junctional complexes with each other. After the second day of culture, most viable trout and catfish hepatocytes were present within the groups of cells. This finding was similar to that observed previously with mouse hepatocytes (*17*), which, when found within groups, displayed greater survival characteristics and were morphologically more similar to the in vivo hepatocytes than cells not in groups.

Both trout and catfish hepatocytes displayed a rapid decline in P-450 and B-5 activities during culture. Despite this decline, measurable amounts of these 2 enzymes were detectable after 8 days of culture. A similar pattern of decreased activities has been observed in rat and mouse liver cell cultures (*28, 29*). This decline may be the result of loss of constant hormone stimulation or other blood-borne factors that influence the hepatocyte in vivo but are absent in vitro. The inclusion into the medium of various glucocorticoid hormone mixtures (*28*), as well as nicotinamide (*30*), and phenobarbital (*31*), has been reported to maintain the cytochrome P-450 activity levels at or near the in vivo measurements for longer periods in cultured mammalian hepatocytes. Although we observed a decrease in the trout and catfish liver cell activities of cytochromes P-450 and B-5, trout hepatocytes were still able to metabolize AFB$_1$ (a mixed function oxidase-dependent reaction) to a DNA damaging form (as measured indirectly by UDS) after 4 days of primary culture. The induction of UDS by AFB$_1$, however, was evident only in its highest concentration examined, which suggested the loss of the required mixed function oxidase activity to metabolize the lower AFB$_1$ concentrations.

Both direct-acting (MNNG) and indirect acting (DMNA, AFB$_1$, and BP) carcinogens induced UDS in the 2 teleost liver cell systems. Although quantitative comparisons in the ability of chemical carcinogens to induce UDS in rodent and teleost liver cells are difficult, our results suggest that the latter cells show a qualitatively lower UDS inducibility. The temperature (25° for the teleost in contrast to 37° C for the rodent liver cells) may influence the rate of carcinogen metabolism, the kinetics of carcinogen entry into the cells, the rate of DNA repair, and the rate of [^{3}H]dThd uptake.

Trout hepatocytes appeared to maintain their ability to repair carcinogen-damaged DNA up to 4 days of primary culture. The amount of UDS induced by MNNG was similar after 4 hours, and 2 and 4 days of culture. The observed decrease in UDS induction by AFB_1 after 2 and 4 days of culture in the trout cells may be related to a decrease in the ability of the cells to metabolize the AFB_1, rather than an inability of the cells to repair the damaged DNA.

In this study, we developed methodology for the isolation and short-term culture of trout and catfish liver cells. These cells have been characterized morphologically and functionally during primary culture. Trout and catfish liver cells can activate indirect-acting carcinogens to DNA damaging forms as measured by the induction of UDS. The catfish and trout liver cell models may provide a valuable tool for the in vitro study of chemical carcinogenesis in the teleost.

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Primary Cell Cultures From the Teleost, *Cyprinodon variegatus*: Culture Establishment and Application in Carcinogen Exposure Studies[1, 2]

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ABSTRACT—Methods were developed for aseptic maintenance of *Cyprinodon variegatus* fry for extended periods. Preliminary studies indicated that under optimum conditions sterile embryos develop normally for a sufficient time to function as carcinogen-teratogen assay systems. An embryo-primary cell culture technique was developed that incorporates, in a single system, certain characteristics of both intact embryos and primary cell cultures and allows simultaneous observation of the effects of carcinogens on the whole organism and primary cell monolayers. The effective use of these systems provides one the opportunity to study the effects of carcinogens on teleosts at the cellular and organismic level. — Natl Cancer Inst Monogr 65: 175–178, 1984.

As the quantity and diversity of potentially carcinogenic chemicals entering the aquatic environment continue to increase, so does the need for the development of new monitoring techniques and procedures that are reliable, rapid, and cost efficient. In recent years, the teleost, *Cyprinodon variegatus,* has been used extensively in toxicology and carcinogen research (*1–7*). In addition to evaluating the usefulness of *C. variegatus* as a test organism for long-term chronic exposures to carcinogens, we have been developing in vitro techniques to study the effects of carcinogens on this species at the cellular level.

Here we describe 2 interrelated techniques currently under development in our laboratory in which we utilize *C. variegatus* tissues. In the aseptic embryo technique, *C. variegatus* embryos are maintained in a sterile environment for extended periods, and, with the embryo–primary cell culture procedure, one can perform carcinogen analyses on a basically intact animal and a primary cell culture in the same system.

MATERIALS AND METHODS

Aseptic embryo preparation.—Egg development was induced by ip injection of 0.05 ml human chorionic gonadotropin (Sigma Chemical Co., St. Louis, Mo.; Stock No. CG5) to female *C. variegatus.* Specimens were injected every other day for a maximum of 3 injections. To harvest the eggs, we killed the females or milked the eggs into a petri dish containing artificial sea water (Rila Marine Mix, 8–12 parts/thousand; Rila Marine Mix, Teaneck, N.J.) by applying pressure along the lateral surfaces of the gravid female. For fertilization, macerated testes were placed into a petri dish containing the eggs. Alternately, we obtained eggs by placing gravid females in a breeding chamber with a male. The fertilized eggs were harvested from a glass egg tray placed beneath the chamber. However, the breeding chamber method did not allow the precise timing of fertilization that could be achieved when the females were killed.

The fertilized eggs were maintained in a petri dish with 2 daily changes of artificial sea water. On the third or fourth day, the eggs were washed with several changes of artificial sea water and, under a laminar flow hood, placed in a petri dish containing sterile Hanks' Balanced Salt Solution containing 5% antibiotics (antibiotic–antimycotic mixture; Grand Island Biological Co., Grand Island, N.Y.). This salt solution was changed three times before the eggs were transferred to a fresh petri dish containing sterile medium. The medium consisted of 9 ml of Leibovitz's L-15 medium plus 1 ml of antibiotics. With sterile forceps or sterile wide-mouthed Pasteur pipettes, the eggs were transferred to a second petri dish containing sterile medium and incubated overnight at 37° C. The eggs were then placed in a fresh dish of sterile medium and incubated until all were dechorionated.

Aseptic embryo technique.—For each experiment, 5 to 15 embryos prepared according to the aseptic embryo procedure were placed into 150-cm^3 plastic flasks (Corning Glass Works, Corning, N.Y.) containing 50 ml of sterile medium. The embryos were maintained at 25° C and examined microscopically on a daily schedule. Experiments were conducted in which embryos were maintained in the basal L-15 plus various combinations of supplements (table 1).

Embryo–primary cell culture technique.—One embryo prepared according to the aseptic embryo procedure was

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TABLE 1.—*Survival times of aseptic embryos*

Culture conditions[a]	No. of expt	Average No. embryos/expt	Average survival time, days
L-15 + 1% antibiotics	1	7	7
L-15 + antibiotics + supplements	4	9	13
L-15 + 1% Abs + supplements + Tetramin tropical fish food	1	10	18
Sterile artificial sea water + sterile *Artemia* eggs[b]	1	2	10
Sterile artificial sea water + 1% antibiotics + Tetramin tropical fish food	1	10	16

[a] Antibiotics were from Grand Island Biological Co. (Cat. No. 6005240): pencillin, 100 IU/ml; Fungizone, 0.25 μg/ml; streptomycin, 100 μg/ml. Supplements were 1% L-glutamine and 10% fetal calf serum.

[b] Eggs were autoclaved before feeding.

placed in each well of 24-well Linbro plates with a sterile wide-mouthed Pasteur pipette, and 4 drops of growth medium was added to each well. The low volume of medium facilitates the attachment process. The developing embryos were incubated at 25° C and observed daily until they were attached to the wells (usually 2–3 days). The growth medium consisted of 10% fetal calf serum in L-15 plus 1% antibiotics and 0.2 m*M* L-glutamine. When the embryos became securely attached, 1 ml of growth medium was added to each well. Throughout the experimental period, the growth medium was changed when necessary, but not more often than once every 2–3 days.

RESULTS

Aseptic Embryo Technique

Our initial experiments indicated that *C. variegatus* embryos could be maintained for up to 18 days under various aseptic conditions (table 1). Fry that were fed autoclaved, particulate food survived longer than those that were not fed. Cannibalistic behavior was observed when large numbers of individuals were placed in the same flask. It was determined that this behavior did not occur if the population density was kept below 1 individual/10 ml of medium.

Embryo-Primary Cell Culture Technique

With 75–80% efficiency, the embryos became attached within 2–3 days, and a mixed population of cells began migrating from the attached region of the embryo (fig. 1). As cell migration continued, the attached region of the embryo became progressively disorganized (fig. 2). Figure 3 illustrates 3 of the morphological types of migrating cells commonly observed: fibroblast-like, pigmented, and ovoid shaped. The pigmented cells often develop interconnecting processes (fig. 4). Frequently, the pigmented cells formed extensive interconnecting networks.

These systems have been maintained for as long as 30 days, and, although the attached area of the embryo may become severely disorganized after this period, the unattached region remains intact and appears to function normally (e.g., the heart continues to beat, limited movements occur, and feeding activity is commonly observed). When the embryo becomes detached from the well or is removed after a monolayer has formed, cells commonly die, as evidenced by rounding and changes in the appearance of the monolayer. This would not be considered unusual in primary explant cultures of this type.

DISCUSSION

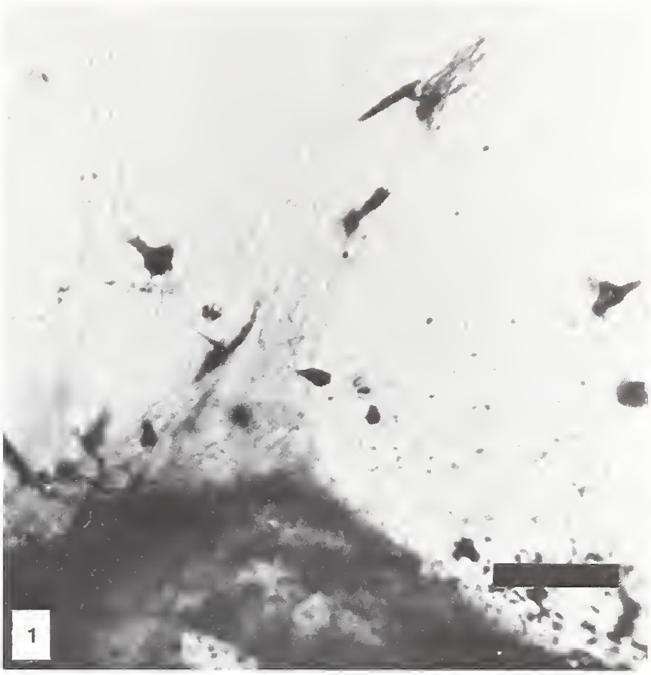
Because of the widespread use of *C. variegatus* in studies on the effects of toxic and neoplastic agents on organisms in the aquatic environment (*3–7*), the need to develop in vitro techniques to correlate with and extend the value of this whole animal system is obvious. The development of such techniques provides a more complete arsenal of tools involving one species that can be used in the aquatic environment comparable to the murine systems already available.

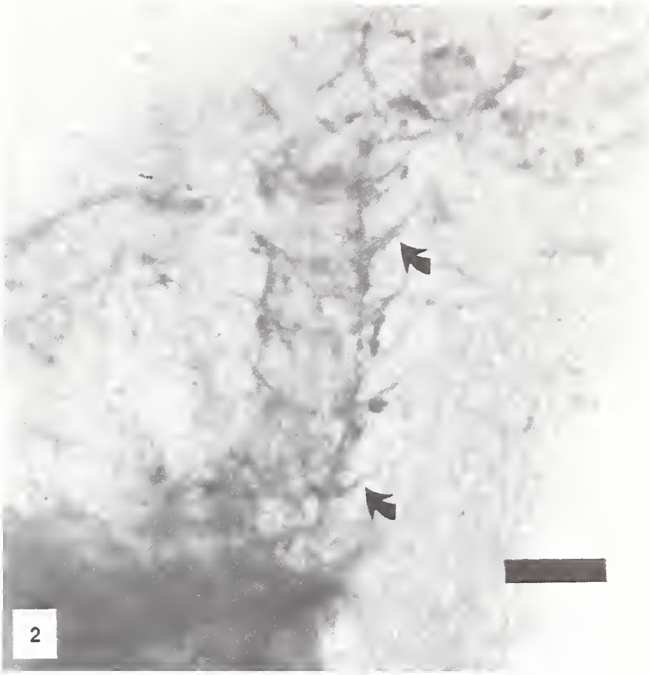
A major advantage of the aseptic embryo technique is that it provides us with an opportunity to study the effects of a contaminant on the organism during its highly susceptible embryonic period entirely free of any influence by bacteria or other organisms normally present. Thus the system should be particularly valuable in carcinogen studies related to enzyme induction and the pathways by which procarcinogens are metabolized into active carcinogenic agents.

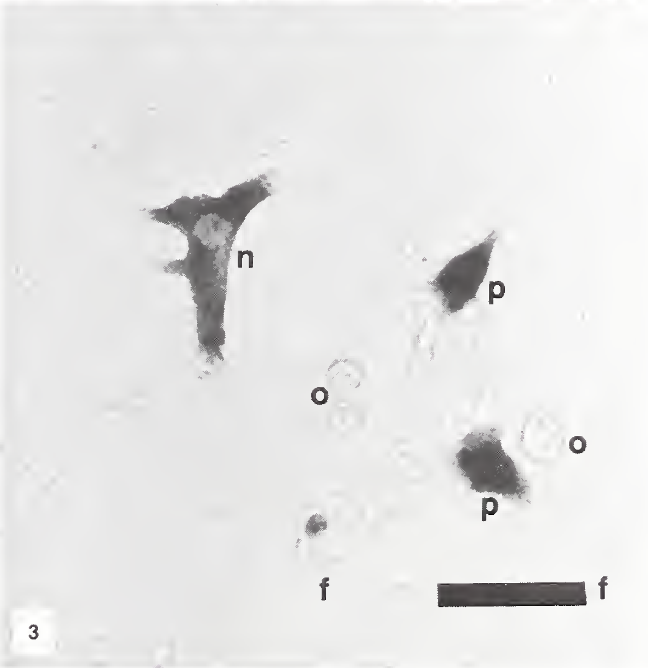
Although the survival times already attained are sufficient for many of the studies anticipated for this system, it is reasonable for one to assume that continued investigation of the system will lead to the development of conditions that will allow normal development of the organisms to an adult state.

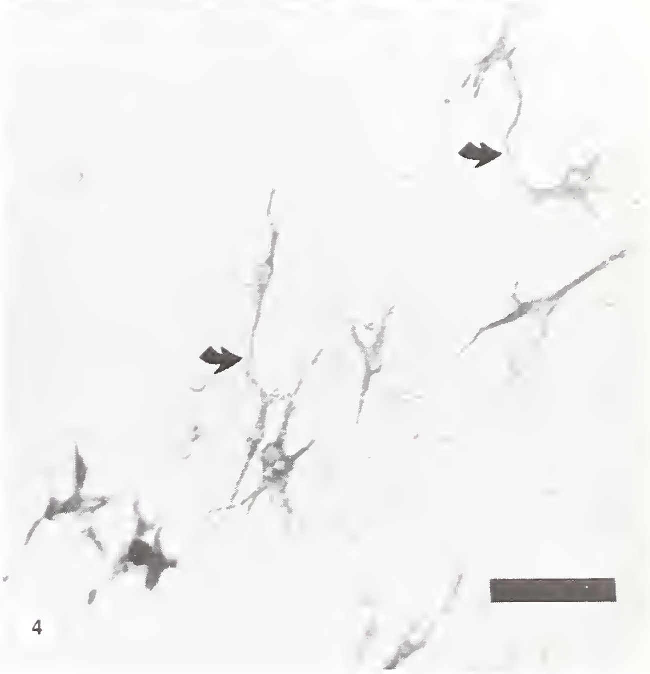
Laale and Lerner (*8*), in a recent review, indicated a need for continued research on teleost teratogens and suggest that these studies must involve the determination of specific metabolic targets. They state that this will require detailed studies of the responding embryos at all levels of organization. Obviously, the present system, in which embryonic development in both contaminated and uncontaminated sterile environments can be carefully observed, provides an excellent system for such ichthyoteratologic studies as well as general studies of teleost embryonic development.

Because the embryo–primary cell culture technique blends the use of an aseptic embryo with cell cultures into one system, it is possible for one to observe simultaneously the effects of a specific carcinogen on an intact organism and primary cell cultures from the same organism. The technique provides numerous advantages. It provides better genetic continuity, i.e., the cells of the primary cell culture can be expected to be more similar, genetically, to the cells of the intact organism than to cells of an









Photomicrographs were taken with bright field optics on an inverted microscope.

FIGURE 1.—Typical appearance after 3 days of culture. Mixed populations of cells migrate from the attached region of embryo. Bar = 50 μm. × 335

FIGURE 2.—Characteristic disorganization of the attached region of the embryo after 2 wk in culture. Most of the cells of the caudal region of this embryo have migrated away from the embryo leaving a partially intact vertebral column (*arrows*). Bar = 100 μm. × 134

FIGURE 3.—Morphology of migrating cells; f = fibroblast-like; o = ovoid-shaped; p = pigmented; and n = pigmented with extended processes. Bar = 50 μm. × 402

FIGURE 4.—*Note* the interconnecting processes of the pigmented cells (*arrows*). Bar = 50 μm. × 335

established cell line. The major portion of the embryo remains intact with normal organ arrangement and function, and the system can be removed for histological examination. Also, the carcinogens make direct contact with the various cells of the system in a chemically defined medium. Not only does the system provide for exposure of the organism during the highly susceptible embryonic development period, but the embryo likely conditions the medium and brings about increased viability and differentiation in the cells of the primary culture. Such conditioning factors have been reported (*9, 10*), and a conditioning phenomenon is consistent with the observations in the present study that the cells in the primary cell culture region change their appearance and behavior after the intact portion of the embryo becomes detached or is removed. Finally, the system bridges the gap between in vitro systems, such as established cell lines, and in vivo systems currently in use.

The technique may be used for a variety of tests and assays, such as: 1) tests for toxicity, teratogenicity, and cell transformations, 2) karyologic alterations, 3) detection of antigenic or enzymatic changes, 4) metabolic activation–co-cultivation, 5) effects on macromolecule synthesis (collagen, mRNA, etc.), and 6) viral genome activation.

Factors that may be considered limitations are: 1) The embryos do not contain the normal microbial flora of their septically reared counterparts; 2) the feeding mechanisms of the cells, and, to some degree, the intact embryos are different from normally reared embryos; and 3) the restricted embryo movement and partial disorganization may bring about metabolic differences as well as other subtle effects. However, even with these limitations, the model provides a useful system that should be reproducible, standardizable, and easily comparable to in vivo systems. Laale (*10*) achieved 40-day survival times with a similar system in which blastoderm isolates from the zebra fish, *Brachydanio rerio,* are used, so it is reasonable that we assume that equal or greater survival times can ultimately be obtained with the *C. variegatus* system. This should provide one adequate time to perform various assays. It is noteworthy here that Hillebert et al. (*11*) obtained multilayered foci in an established cell line from the sheepshead, *Archosargus probatocephalus,* exposed to benzo[*a*]pyrene after only 12 population doublings.

In summary, these interrelated techniques allow the evaluation of the effects of carcinogens on *C. variegatus* cells and embryos maintained in a sterile environment. Together, they bridge the gap between subcellular rapid assessment techniques and whole-animal exposure methods. Furthermore, they provide additional tools comparable to the traditional murine systems and extend the value of *C. variegatus* as a model system for laboratory studies of the effects of pollutants in the aquatic environment.

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Culture of Embryos and Cells From Platyfish and Swordtails for Carcinogenicity Testing and Studies on Melanoma Formation[1]

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ABSTRACT—We introduce inbred strains of platyfish and swordtails as well as their hybrids as laboratory animals for carcinogenicity testing. We especially propose to take advantage of the platyfish-swordtail melanoma system, which permits the breeding of animals with high susceptibility to carcinogen-induced melanoma. Based on our present view that, in melanoma formation, such fundamental processes as the determination and differentiation of a specific cell type are affected, we suggest the use of embryos and embryo-derived cell cultures. In both systems, all stages of pigment cell differentiation can be followed and screened for abnormal development. The in vitro culture of the embryos of the viviparous fish and the establishment of cell cultures are described. — Natl Cancer Inst Monogr 65: 179–183, 1984.

When testing chemical and physical agents for carcinogenicity, one has to consider that the genetic constitution of the test animal might greatly influence the response to a specific carcinogen. Scientists have repeatedly pointed out that the genetic heterogeneity of most fish populations in nature may make it difficult for one to interpret the experimental results obtained with those fish. Therefore, several authors have suggested the use of highly inbred strains or, even better, the use of naturally occurring or experimentally produced clones of homozygous fish as have several participants in this Symposium. To evaluate the potential hazard of a certain agent, one should compare the data obtained from different strains or clones of the same type of fish.

A system which provides both homozygous strains and various genotypes is represented by the 8 species of the genus *Xiphophorus,* commonly known as platyfish and swordtails. These fishes have been used as laboratory animals for more than 50 years. They are small viviparous fish which can easily be bred in large quantities, with a generation time of less than 6 months, have an ovarian cycle of 4 to 5 weeks, and give birth at regular intervals to large litters of young (*1, 2*). Approximately 7 days after a brood has hatched, a new set of oocytes develops into mature eggs and can be fertilized. The gestation period lasts about 22 days. Many inbred strains of platyfish and swordtails are available (*3*), and all species and strains can be crossed by natural or artificial insemination (*2, 4*). The distribution of chromosomes in the various hybrid constellations can be followed by isoenzyme markers (*5*). Besides providing excellent material for carcinogenicity testing in general, these fish can be used as test animals in a more specific way if one takes advantage of the platyfish–swordtail melanoma system. In our introduction of this system, we present our views on the principles underlying the genetically based formation of melanoma and describe, in addition, the in vitro culture of early fish embryos and the establishment of cell cultures derived from embryos. These procedures are the basis for the use of embryos and cultured cells in further studies on melanoma formation and in carcinogenicity testing.

THE MELANOMA SYSTEM

Since the early work of Gordon in 1931 (*6*), the formation of melanoma in platyfish–swordtail hybrids has been the subject of many genetic and cytological investigations [for reviews, *see* (*7–10*)]. In most instances, the parental species of the hybrids are the platyfish, *Xiphophorus maculatus,* and the swordtail, *X. helleri.* The platyfish is marked by black spots which form specific patterns at various body sites. These spots are built up by huge melanin-containing pigment cells, the macromelanophores, which differ significantly from the normal melanophores of the skin. They are inherited by a single Mendelian factor, the *macromelanophore gene.* In contrast to the platyfish, the swordtail does not develop macromelanophores. When the platyfish is crossed with the swordtail, the resulting F_1 hybrids develop many more macromelanophores than the platyfish parent. When such an F_1 hybrid is backcrossed to the swordtail, all backcross hybrids that inherit the macromelanophore gene produce even more of these cells, which results in melanoma formation. This formation is due to a loss of macromelanophore-specific regulating genes by the replacement of platyfish chromosomes with those of the swordtail. Besides these *nonlinked* regulating genes, the platyfish also carries a *linked* regulating gene which cannot be outcrossed because it is located on the same chromosome as the macromelanophore gene. Several alleles of the linked regulating gene differ in their influence on the location, amount, and size of the macromelanophore spots. Depending on which allele of the linked regulating gene and how many of the nonlinked regulating genes are present or absent in the genome, the

ABBREVIATION: PBS = phosphate-buffered saline.

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individual fish produces only the typical platyfish spots, benign melanomas, or malignant melanomas.

By transplantation experiments, Humm and Young (*11*) established that macromelanophores and normal melanophores originate from pigment cell precursors which develop from the neural crest during early embryogenesis. According to Tavolga (*1*), the neural crest can be identified in embryos of stage 11 as a small group of cells on each side of the neural tube. During the following stages, the pigment cell precursors are released from the neural crest and normal melanophores appear on the skin in a wave, starting from the anterior of the embryo and moving progressively to the posterior. Depending on the genotype, macromelanophores develop during either late embryogenesis or further development of the young fish. In fish of some genotypes, the first macromelanophores appear at stage 20 (fig. 1A) and cover almost the entire body before the embryos reach the birth stage (fig. 1B).

Based on the results of genetic (*12*), cytological (*13*), and biochemical (*14*) investigations and on the results of a gene transfer experiment (*15*), we developed a model for the possible functions of the macromelanophore gene and its regulating genes (*10*). According to this model, the macromelanophore gene acts by determining the pigment cell precursors and shifting these cells into a hyperplastic state in which they tend to proliferate before differentiating into macromelanophores. A closely linked regulating gene is thought to restrict the number of precursor cells in which the macromelanophore gene can become active. As a result, only a limited number of cells enter the macromelanophore pathway. The proliferation and differentiation of the determined cells is controlled by several nonlinked regulating genes. Any loss of regulating genes by mutation or outcrossing results in early appearance and overproduction of macromelanophores. Thus melanoma formation in fish can be considered a genetically based disorder in the determination and differentiation of this specific pigment cell type.

For a better understanding of these processes, the development of macromelanophores should be studied from their initiation. This can be done in embryos from fish of different genotypes as well as in cell cultures derived from these embryos.

IN VITRO CULTURE OF EMBRYOS

The following procedure has been developed for the culture of platyfish and swordtail embryos from an early stage, i.e., as early as the pigment cell precursors start to migrate from the neural crest. For this purpose, we used embryos at stages 9 to 12 (fig. 2A, B). The procedure has already been published in a German journal of limited circulation (*16*). A more comprehensive description of the underlying experiments will soon be published in English (Vielkind U, Haas-Andela H, Vielkind J: Manuscript in preparation).

The embryos are isolated under aseptic conditions from pregnant females 11 days after the last brood has hatched, i.e., 4 days after fertilization of the eggs (*1*). They are separated from the ovarian tissue and washed in sterile Dulbecco's PBS (diluted 8:3). Each embryo is then placed into a test tube containing 2 ml of sterile PBS containing 100 IU penicillin and 100 μg streptomycin/ml. The embryos are incubated at 27–29° C in the dark until they have reached the stage of birth (stage 26). With PBS as a culture medium, early embryos develop normally only until stage 20. Thereafter, development is drastically disturbed, and only 10% of the embryos reach the birth stage. To improve the culture conditions, we prepare the PBS with "conditioned tank water," which is water from an aquarium in which adult fish have been kept for at least 4 weeks. That the use of conditioned tank water is essential for a proper development of embryos has already been reported by Humm and Young (*11*), who cultured embryos at stage 20 and older. Nevertheless, only 25% of the embryos show normal development and survive until the stage of birth in PBS prepared with conditioned tank water. However, supplementation of this medium with low-molecular weight organic compounds yields a much higher rate of survival. The best results are obtained with PBS prepared with conditioned tank water to which 2 ml of chick embryo extract, 250 mg glucose, and 250 mg casein hydrolysate/liter has been added. With this medium, about 80% of the embryos survive, although development is delayed for up to 12 days compared with normal development in vivo. One need not strip the chorion of the embryo mechanically or enzymatically, inasmuch as during in vitro culture the

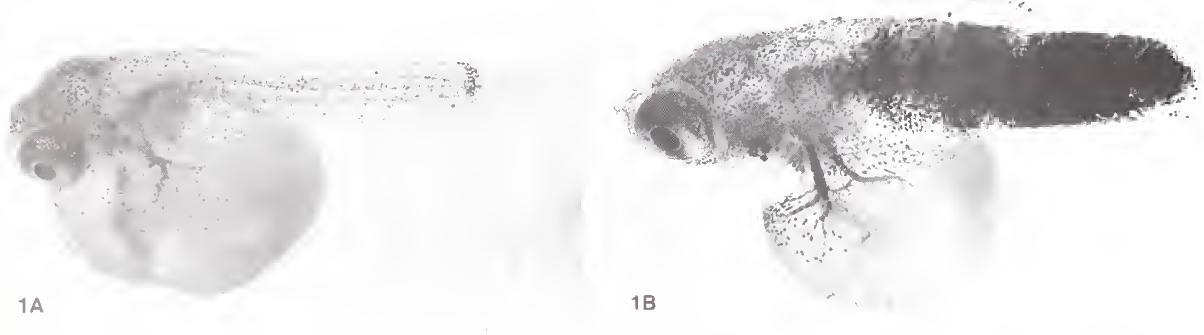

FIGURE 1.—Embryos of a platyfish mutant. A) Stage 20, a few macromelanophores in the caudal peduncle; B) stage 25, many macromelanophores covering almost the entire body.

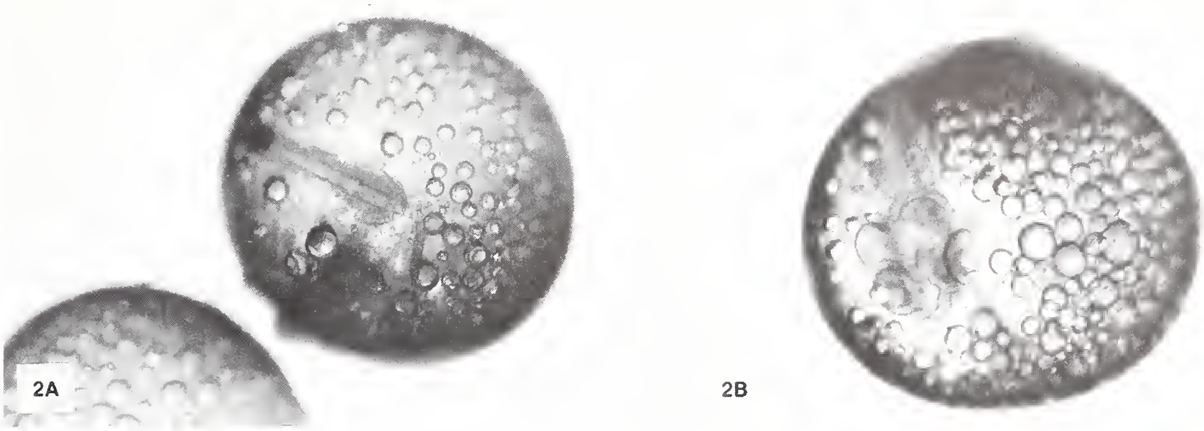

FIGURE 2.—Platyfish embryos. A) Stage 9; B) stage 12.

chorion usually bursts at about stage 20 and is stripped by the embryo through vigorous tail movements.

At the stage of birth, many of the in vitro-cultured embryos have not resorbed all the yolk. The yolk sac is usually still large because it is filled with liquid. Therefore, a critical step is the release of the embryos from the culture medium into the tank water. Most of the embryos do not survive an immediate transfer, probably due to the difference in the osmolarity between the medium and the tank water. To overcome this problem, we placed the embryo and the culture medium into a dialysis bag, which is hung into conditioned tank water at 25–27° C for approximately 24 hours. During that time, the yolk is usually completely resorbed and the yolk sac disappears. The embryos are then released into conditioned tank water. Initially, the water level has to be as low as 2–3 cm, so that the young fish can easily reach the surface to inflate the swim bladder. The water level is then gradually raised during the next 2 weeks. Supplementation of the water with peat moss extract keeps the pH slightly acidic and avoids overgrowth by microorganisms. The young fish are fed daily with live nauplii of the brine shrimp *Artemia salina* and with powdered Tetramin dry food. If the described procedure is followed, almost all the embryos survive the transfer to the tank water and can be grown to adult fish.

In total, about 70% of the embryos survive the in vitro culture and the transfer to the tank water. This is only 10% less than the usual survival rate of newborn fish. Most of the embryos grow to perfectly normal and fertile fish. Only 2% of the adults show malformations, such as curvature of the spine (fig. 3A), abnormal body shape (fig. 3B), atrophic eyes, or an indentation behind the head. These malformations are identical to those that occur in less than 1% of fish born normally. The increased incidence of these malformations is probably due to mechanical injury during manipulation of the embryos at an early stage of development or during stripping of the chorion at later stages.

ESTABLISHMENT OF CELL CULTURES

The following procedure has been developed for the start of primary and secondary cell cultures which contain a high percentage of pigment cells. For this purpose, we used embryos that have already reached stage 20 (11 days after fertilization). By this stage, many pigment cell precursors have developed and migrated to their final location in the skin, and normal melanophores appear all over the body. A detailed description of the procedure and of the underlying experiments has been published (*17*).

All solutions used for the establishment and maintenance

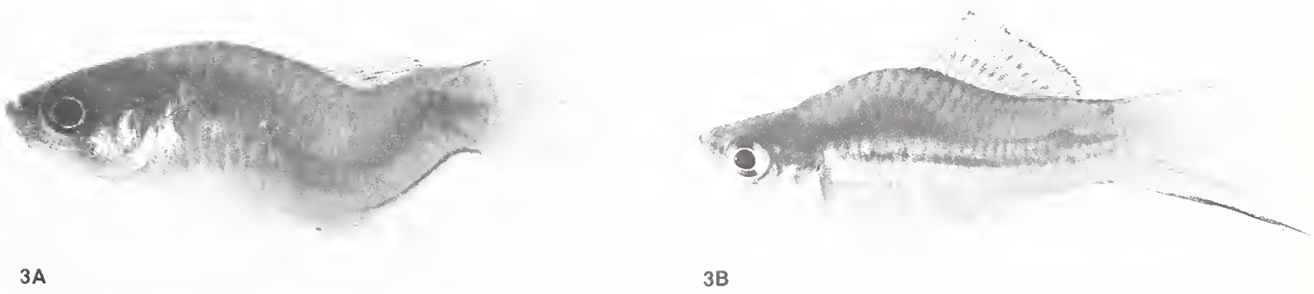

FIGURE 3.—Adult swordtails developed from in vitro-cultured embryos. A) Female with curvature of the spine; B) male with abnormal body shape.

of the cell cultures contain 100 IU penicillin, 100 μg streptomycin, and 2.5 μg amphotericin B (Fungizone) per ml. The embryos are isolated under aseptic conditions from pregnant females 18 days after the last brood has hatched. They are washed in sterile undiluted Dulbecco's PBS, separated from the yolk sac, and cut into small pieces. After the tissue is washed several times in sterile PBS, it is disintegrated into single viable cells according to the following procedure: The pieces are incubated in Ca^{2+}- and Mg^{2+}-free Hanks' salt solution containing 0.01% trypsin and 0.2 *M* EDTA. The pH is adjusted to 7.0, and the solution is incubated at 4° C under constant stirring. After 1 hour, it contains predominantly damaged cells and therefore is removed and replaced by fresh trypsin solution. After an additional 2 hours, the solution contains viable cells. It is decanted, stored on ice, and replaced by fresh solution. This is done three times for 2 hours. To each cell suspension, 20% serum is added immediately so that trypsin activity is inhibited. Finally, the dispersed cells are harvested by low speed centrifugation. This procedure yields a maximum of 85% viable cells, as we have judged by trypan blue staining.

Primary cultures are started with a minimum of 3×10^6 cells/ml medium. Among the commercially available media commonly used for mammalian cell cultures, Medium 199 proved to be the best, followed by Diploid Growth Medium, and Eagle's Minimum Essential Medium and Basal Medium Eagle. Contrary to other reports, one need not dilute these media for fish cell cultures (*18*). The cells are cultured in a closed system at the optimal temperature of 27° C. At this temperature, the stabilization of the pH at 7.2 is a critical point because the bicarbonate content of the media is adjusted for use at 37° C, i.e., the temperature required for mammalian cell cultures. To overcome this problem, which would result in frequent replacement of the medium, we supplement the medium with 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. This organic buffer keeps the pH constant even over long periods. As a result, the cells grow significantly better and usually form a confluent monolayer about 4 days after seeding. For initiation of secondary cultures, the monolayer cells are dispersed with trypsin-EDTA for a maximum of 5 minutes at 24° C. After the addition of serum, the cells are harvested by centrifugation, and subcultures are started with 3×10^5 cells/ml medium. The medium is changed every 5 days, and the cultures are split after a confluent monolayer is formed.

The embryo-derived cell cultures contain several cell types. In primary cultures, various proportions of epitheloid, fibroblast-like, and pigment cells are present (fig. 4). Immediately after seeding, the pigment cells are in the low-differentiated stage of poorly pigmented, spindle-shaped melanocytes, but they tend to differentiate rapidly through dendritic melanocytes into heavily pigmented, stellate melanophores of both types. Thus primary cultures of embryonic cells provide useful material for the study of the differentiation of pigment cells in vitro. In secondary cultures, epitheloid and fibroblast-like cells predominate, whereas the pigment cells are eliminated stepwise from subculture to subculture. This is due to their tendency to differentiate rapidly into stages in which they are no longer able to divide. We hope to increase the percentage of pigment cells in secondary cultures by modifying the culture conditions in such a way that the propagation of pigment cells is favored over that of other cells.

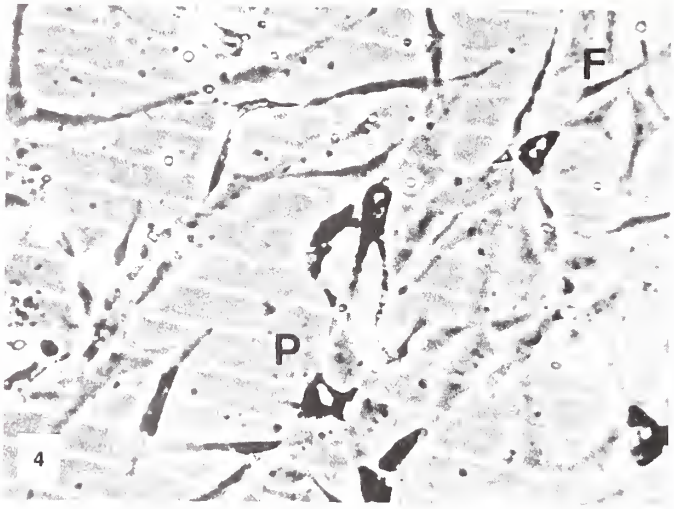

FIGURE 4.—Primary cell culture derived from a platyfish embryo at stage 20. P = pigment cells; F = fibroblast-like cells.

CONCLUSIONS

The in vitro culture of embryos and the establishment of cell cultures containing pigment cells and their precursors offer the possibility of studying the development of pigment cells simultaneously in the living embryo and in cultured cells. In this way, it should be possible for one to explore the initial steps in melanoma formation, i.e., in the disturbance of determination and differentiation of macromelanophores. Furthermore, embryos and cell cultures from fish of defined genotypes can be used in the testing of physical and chemical agents for their carcinogenic potential. Some of these agents might modify the determination and differentiation of macromelanophores by interfering directly with these processes; others may act by causing mutations of the genes controlling these processes. The latter mechanism can be studied as follows: Ahuja et al. (*19*) have shown that fish of different genotypes differ significantly in their susceptibility to various types of neoplasia. In some hybrids which do not develop melanoma spontaneously but which have a certain genetic disposition, melanoma formation can be induced by treatment with chemical or physical mutagens (*19, 20*). Based on this finding, Anders et al. (*21*) developed a strategy for breeding test animals with high susceptibility to carcinogen-induced melanomas. These animals lack all nonlinked regulating genes but carry a macromelanophore gene complex containing a strong linked regulating gene. Thus embryos and cell cultures of this genotype should provide excellent test systems for the detection of potential carcinogens because the somatic mutation of this single regulating gene in 1 pigment cell precursor should result in the formation of several macromelanophores in the embryo or cell culture, respectively.

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Session III

Maintenance of Fish in the Laboratory

Chairman: **Herman Kraybill, Ph.D.**

Physiological Responses of Experimental Fish to Stressful Conditions[1]

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ABSTRACT—Common stress factors in the laboratory aquarium can include extremes of pH, temperature, or other factors, such as the accumulation of intermediates of nitrification. Each aberration from optimal conditions has a direct physiological effect on fish that can cause death in a variety of ways. The stress effect in fish leading to infectious diseases is a common physiological manifestation of some common biochemical change, probably hormonal, which may compromise the immune system and lead to a decreased disease resistance and ultimately death. Minimization of stress by careful attention to basic fish culture practices is perhaps the best way one can ensure successful laboratory experiments. — Natl Cancer Inst Monogr 65: 187–193, 1984.

In broad terms, the physiological response of a fish to a stressful condition could include immediate death from cardiovascular collapse to a delayed death from bacterial disease as a result of a stress-induced immunological failure. Many physiological changes occur between these extremes.

The title of this paper will evoke a variety of responses from individuals, depending on their previous experience with keeping fish under laboratory conditions. The term "laboratory aquarium" itself can evoke various opinions among aquarists on how best to keep fish in regard to size of aquaria, filtering systems, and general maintenance. Similarly, the type of experimental fish could vary depending on size, hardiness, susceptibility, or resistance to a particular disease agent or responsiveness to a carcinogen. Generally, "stressful conditions" are well-known to the experienced aquarist who often has a backlog of experiences that would indicate which species of fish can tolerate various stresses. Lastly, physiological changes are perhaps the least obvious to the fish culturist because discrete changes unaccompanied by clinical signs or behavioral changes would be detectable only by clinical pathological examination.

Our purpose here is to review pertinent basic concepts concerning environmental control of fish culture systems and minimization of stress and deleterious physiological responses. We also review the origin of physiological stressors in the laboratory aquarium and the response of fish to them.

THE STRESS SYNDROME IN FISH

The term "stress" has a number of definitions. The most satisfactory definition for the content of this paper is that of Esch and Hazen (*1*), who define stress as the effect of any environmental alteration or force that extends homeostatic or stabilizing processes beyond their normal limits at any level of biological organization. We will also consider the stressor to be any environmental change that affects the organism such that a physiological response is required.

The stress response of fish to a wide variety of stressors in many ways is similar to that described for mammals as the general stress syndrome (*2*). Basically adrenocorticotropic hormone from the pituitary controls the release of catecholamines (epinephrine and norepinephrine) and corticosteroids. In fish, these stress hormones are released from chromaffin and interrenal tissues, which are analogous to the adrenal tissues in mammals. Among the physiological changes caused by the release of these hormones are dilation of the gill filamental arteries, an increase in stroke volume of the heart, increased glycogen metabolism, and depression of the immune response.

In the wild, the stress response is generally short-term and advantageous to the fish. When a fish needs additional energy for a short-term emergency, dilation of the filamental arteries and an increase in the heart's stroke volume will increase the uptake of oxygen. This increase coupled with that in the breakdown of muscle glycogen increases the available energy.

Fish kept in the laboratory are also exposed to factors that evoke the stress response; however, they are often exposed for longer periods than fish in the wild. For example, handling and transporting will cause the stress response. Also, fish in the laboratory are often exposed to poor water quality conditions, but unlike fish in the wild, they cannot alleviate the stress caused by the poor water quality by moving to another area. Consequently, fish in the laboratory are often exposed to water quality conditions that act as stressors for prolonged periods.

Within physiological limits, fish can acclimate to many factors that initially will cause a stress response. However, if the stress is beyond these limits and is maintained for prolonged periods, the health of the fish can be seriously affected. For example, although increased blood flow through the gills increases the availability of oxygen, over prolonged periods of stress, such as when fish are being transported or during severe handling, the increased blood flow can cause serious osmotic problems. The increased blood flow will lead to increases in the uptake of water and in salt loss at the gills in freshwater fish. A simple remedy described by Long et al. (*3*) that has dramatically reduced mortality during the handling and hauling of salmon smolts has been to decrease the osmotic difference between the water and the fish's blood by adding about 3 parts per thousand of salt to the fish's water.

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Another serious problem caused by the stress response is its depression of the immune response in fish. Therefore, the longer fish are stressed, the greater the chance for the outbreak of disease. Various aspects of problems caused by disease as well as the specific effects of various water quality parameters are discussed in the following sections of this paper. For anyone interested in learning more about the stress response in fish, an excellent source is the book "*Stress and Fish*" (*4*).

ENVIRONMENTAL STRESS AND DISEASE

A basic premise in fish culture is that various protozoal, viral, or bacterial pathogens are likely to be present in a fish culture system. Many of us have repeatedly observed that clinically apparent diseases will often follow a stressful period caused by various environmental changes (*5*). Therefore, the maintenance of healthy fish appears to have 2 components: a fish devoid of parasites and an aquatic system with optimal water conditions for the species.

THE LABORATORY AQUARIUM

The maintenance of fish for a short term poses no problem other than the provision of aerated and dechlorinated water in a container of suitable size. Under such short-term experiments, toxic levels of metabolic waste products would accumulate in a few days to weeks, depending on the amount of fish and volume of water. If fish are kept for a longer period, water quality can be maintained by alternative methods. These include frequent water changes without filtration, a flow-through system, and a filtration system.

Many fish can be kept in an aquarium indefinitely without filtration if the water is changed frequently and if attention is given to periodic removal of organic debris. In this most simple method, regular water changes must be made if one is to avoid accumulation of ammonia and other toxic nitrogenous products such as nitrites. An aquarist can determine the frequency of changes by simply not allowing the water quality to deviate from what is considered to be adequate. Ranges for water quality characteristics sufficient for maintaining fish are presented in table 1 (*6*).

The advantage of a simple water replacement system is that it is economical. Because only aerated aquaria are required, expenses are minimal. Disadvantages include the need for continual cleaning and water changes (which disturb the fish), variance of water quality between aquaria, and the need for a consistent source of clean uncontaminated water.

The essential concept of a flow-through system is that water is continually replenished with fresh water. Aeration may or may not be required depending on the number of fish and the degree of surface agitation. Waste water is not recirculated. The advantages of a flow-through system include the avoidance of accumulated waste products, clear water, and consistent water quality values between aquaria. Because of the constant flushing action, spread of parasitic diseases is minimized. Disadvantages include the costs of water that may require heating or cooling. By using a flow-through system for carcinogenicity testing, the researcher can regulate dosages by a delivery system described by Mount and Brungs (*7*).

TABLE 1.—*Water quality for fish culture*

Water chemistry	Acceptable range or upper limit
Acidity	pH 6–9
Total hardness	50–300 ppm (as calcium carbonate)
Alkalinity	At least 20 ppm as calcium carbonate
Oxygen	5–8 ppm
Carbon dioxide	3–10 ppm
Nonionized ammonia	0.02 ppm, upper limit
Nitrite	0.1 ppm, upper limit

Recirculating systems refer to the reuse of water by fish after the water is filtered. In individual holding containers or aquaria, the filter can be external to, or placed in, the aquarium. In general, filtration can be categorized as having a mechanical, biological, and chemical function. Mechanical filtration is the simple entrapment of particulate matter by any substance, such as a fibrous floss, sponges, or gravel. Biological filtration is the oxidation of ammonia to nitrites and nitrates effected by nitrifying bacteria. The aquarist can use a chemical filtration system to accomplish specific desirable changes in water quality. For example, activated carbon will remove various organic molecules including those responsible for color, and ion exchangers can soften water or remove specific cations or anions.

Various types of filters are available. Box-type filters are placed inside an aquarium, and water is circulated by air flow. Such filters can be charged with a floss matrix, carbon, and gravel which act as a substrate for nitrifying bacteria.

Various types of filters, which are located outside the aquarium, are available and can be powered by an airlift but usually are driven by electric-powered impellers.

An undergravel filter is a plate that covers part or the whole of the bottom of an aquarium. Aquarium gravel is placed on the plate to a depth of from 6 to 8 cm. Water is driven through the gravel layer by the action of an airlift located at one or two corners of the filter plate. The gravel bed acts as a mechanical filter and later, with the establishment of nitrifying bacteria, as a biological filter.

Two other types of filters are foam and rapid sand filters. The foam types act as efficient mechanical as well as biological filters. The rapid sand filter is used extensively in swimming pool facilities and larger aquaria and is nothing more than a mechanical and biological filter.

The disadvantage of using a filter system for an individual aquarium is that maintenance of similar water conditions from aquarium to aquarium is difficult. This disadvantage can be overcome with the use of a central filtration system, whereby an individual aquarium continually receives water from a centrally located filtration system. Basically, the recirculated water in each aquarium should be similar in composition. The filter unit can include a settling basin, sand or gravel filters for mechanical and biological filtration, rotating plastic disks or submerged plastic rings for biological filtration, diatomaceous earth filters for removal of fine particulate matter, and UV light for disinfection. Chemical filtration with activated carbon or natural zeolites for ammonia removal are frequently used. Spotte (*8*) published an excellent review of water management in closed systems.

SOURCES OF STRESS IN LABORATORY AQUARIA

Water Source

In flow-through or simple water replacement systems, water characteristics will depend on the municipal water system or well water. In general, the characteristics of water will vary from region to region with respect to hardness, alkalinity, pH, and trace mineral elements. In some instances, water may require partial softening and the addition of buffers for proper pH control. [See (*9*) for formulations for making suitable water.]

Conditioning Period

Whenever a filtration system is used, chemical changes occur in aquarium water that result from the process of nitrification (*8*). When any type of closed aquarium system is initially established, ammonia excreted by fish will accumulate and after a time will be converted to nitrites and eventually to nitrates. This initial conditioning period corresponds to the time during which a population of chemoautotrophic bacteria, such as *Nitrosomonas* and *Nitrobacter*, are forming on the surface of the filtering media. The end of the conditioning period corresponds to the time when nitrites are absent from the aquarium.

Results of experiments done in our laboratory suggest that nitrites are the principal cause of death during the conditioning period (*10*). One can avoid the problem by testing and changing the water regularly and by seeding gravel beds with "conditioned" gravel (10% conditioned gravel). We have found that corner foam filters which have been conditioned are excellent for maintaining fish for extended periods. As nitrification proceeds, the pH of the water may drop if the buffering capacity is low.

Ammonia

In addition to the accumulation of ammonia during the conditioning period, it will also develop if the pH of the water drops to approximately 5.5 or if excessive feed is placed in the aquarium and uneaten feed is not removed. The addition of more fish to an aquarium may exceed the capacity of the filtering system for maximal oxidation of ammonia. Also, some antibiotics and therapeutants can effectively inhibit the action of a biological filter resulting in an accumulation of ammonia and nitrites (*11, 12*).

The toxicity of ammonia depends on the pH of the water. Ammonia exists in 2 forms: nonionized and ionized. The nonionized form is toxic and predominates at higher pH values, whereas the ionized form is nontoxic and predominates at lower pH values.

The physiological effects of ammonia on fish are complex and can be categorized into acute and chronic. In his study on rainbow trout, Smart (*13*) reported that histopathological changes induced by lethal ammonia concentrations were minor. The lifting of the lamellar epithelium observed on the gill was judged to be insufficient to have a significant effect on the water–blood diffusion distance, which suggested that an impaired oxygen uptake resulting from gill damage was not a primary acute toxic action of ammonia. Brockway (*14*) discovered that with an increase in ambient ammonia, the level in blood oxygen is decreased; therefore, ammonia may affect the oxygen carrying capacity of the blood, and presumably death is a result of asphyxiation. However, the work of Fromm and Gillette (*15*) does not support this premise. They suggest that increased blood levels of ammonia in fish subjected to ambient ammonia levels are due to the inhibition of ammonia excretion rather than the inward transfer of ammonia against a concentration gradient. They demonstrated by in vitro studies that an increase in nonionized ammonia to 10 mg/ml had no significant effect on the ability of hemoglobin to combine with oxygen. Lloyd and Orr (*16*), who observed that when rainbow trout were subjected to ambient ammonia the permeability of the gill to water increased, suggested that death resulted when the increase in permeability exceeded urine output. Another possible acute toxic mechanism of ammonia is that increased blood levels interfere with neural function (*17*).

The chronic effects of ammonia include extensive gill pathology which could cause asphyxiation. The incidence of myxobacterial gill disease in conjunction with high ammonia levels suggests that ammonia is an important predisposing stressor to this disease (*18*). Other effects of ammonia that have been suggested are 1) its release in the unprotonated form can raise the pH in cell fluids, 2) it inhibits amino acid catabolism and cellular energy production, and 3) it interferes strongly with certain membrane functions, such as the active transport of monovalent cations (*19*). Finally, the toxicity of ammonia is not only influenced by pH but also by temperature, alkalinity, and oxygen and carbon dioxide levels (*16, 18, 20–22*).

Nitrite

Generally, nitrite toxicities in aquaria are associated with recently established systems during the conditioning period. Nitrite toxicity can also result from the destruction of nitrifying bacteria following disinfection of filters. The direct cause of death is the formation of methemoglobin resulting in the asphyxiation of the fish. Various species of fish differ in their susceptibility to the toxic effects of nitrite. The 96-hour 50% lethal concentration for channel catfish is approximately 7.5 mg nitrite nitrogen/liter (*23*), whereas that for rainbow trout ranges from 0.19 to 0.39 mg nitrite nitrogen/liter, depending on the size of the fish (*24*). We noted that some species of fish appear to be tolerant to nitrites. Fish exposed to nitrites appear listless and refuse food. On postmortem examination blood appears to be rust colored.

Acid Stress

In closed freshwater aquarium systems, pH levels should be kept between 6.8 and 7.8. Maintenance of pH toward neutrality will ensure that any ammonia present will be predominantly in the nontoxic ionized form. In closed freshwater recirculating systems, a pH of below 5.5 will result in cessation of the oxidation of ammonia. Low-pH water can develop in an aquarium by the addition of acidic water, by mistakes in adjustment of the pH, and as a result of biological degradation of organic wastes, especially when water is poorly buffered.

From various reports (*25–27*), it appears that a water pH of between 4 and 5 will have deleterious effects on fish. The basic mechanism of the toxic effect of acid water is unknown, but a few mechanisms have been suggested (*28*). Continuous exposure to low pH water can result in the lifting of surface epithelium from the gill vasculature. This in addition to mucus coagulation can lead to hypoxia and death.

A primary result of low pH to the gill epithelium would also affect electrolyte homeostasis. A decrease in the pH of blood because of acute acid water stress could result from the influx of hydrogen ions across gill membranes. A low water pH level could lower gill calcium levels resulting in the increased permeability of gills to both hydrogen and sodium ions and result in acidosis. This condition may also be caused by the inhibition of excretion of hydrogen and carbon dioxide because of gill damage. Finally, acidic water could lead to decreases in blood pH with eventual overwhelming of blood buffering capacity. The final result in this sequence would be lowered blood pH and then a decreased affinity of hemoglobin for oxygen.

Environmental Contaminants

In the past 25 years, the effects of contaminants on fish have received a great deal of attention. Of the hundreds of contaminants that have been demonstrated to be toxic to fish, the 2 groups that have caused the most problems are synthetic organic compounds and heavy metals. Although these materials are generally more of a problem in the field, they can affect fish in the laboratory. For example, insecticides in food from contaminated glassware or from a contaminated water supply can have deleterious effects on fish. Many of the chlorinated hydrocarbon insecticides have 96-hour 50% lethal concentration values in the low parts per billion, and chronic exposure to parts per trillion of some of these can affect the growth and reproduction of fishes (*29*).

Of the heavy metals, mercury has received the most notoriety because of the public health hazard presented by mercury-contaminated fish. In the laboratory, however, heavy metals such as iron, copper, and zinc generally cause the most problems. Iron is often found in well water in concentrations that are deleterious to fish, and high concentrations of copper and zinc are often associated with pipes and metal valves in water lines.

Heavy metals can stress fish in several ways. The most commonly noted is the formation of mucus on the gill filaments that will inhibit oxygen uptake. Some heavy metals also affect cellular membrane permeability and some, such as cadmium, mimic metal co-factors and affect enzyme activity.

Differences in water quality factors, such as temperature, dissolved oxygen, pH, and alkalinity, affect the toxicity of many contaminants. For example, copper is about ten times more toxic to rainbow trout (*Salmo gairdneri*) in soft water (12 mg calcium carbonate/liter) than in hard water (320 mg calcium carbonate/liter) (*29*). For these reasons water quality factors must be considered in any attempt at assessment of the toxicity of contaminants found in water supply systems. [*See* (*29*) for an excellent reference for the acute and chronic effects of contaminants in aquatic systems.]

The most common contaminant in city water supplies is chlorine. City water can be used in studies involving aquaria; however, the water must be dechlorinated by aeration for 24 hours or by the addition of sodium thiosulfate. In a flow-through system in which chlorinated water is used, dechlorination must be achieved with activated carbon. A combination of the various filters that are available will remove most of the contaminants which present problems to fish.

Temperature

Temperature fluctuations are easily controlled in laboratory aquaria by adjustment of room temperature or by the use of immersion heaters. Behavioral and physiological effects caused by temperature variability have been reviewed in detail (*30*). From the viewpoint of fish as a test animal for carcinogenicity testing, it would seem advantageous for an aquarist to maintain fish at their optimal temperature for growth. The range of temperatures over which fish survive is wide (*31*), and no one fish species can survive over the entire range. Over most of a fish's temperature range, increases in temperature accelerate aspects of metabolism, such as growth and elimination of metabolites. Deaths associated with abrupt temperature changes are most likely caused by cessation of essential enzyme functions associated with respiration. Adaptation to extremes of temperature may involve changes in the amounts of enzymes or the formation of isoenzymes (*30*). Because fish can be acclimated to higher than optimal temperatures, the onset of carcinogen-induced tumors may be accelerated by an increase in the water temperature.

Crowding

The number of fish which can be kept in one aquarium will depend on the water exchange rate in a flow-through system or on the biological filtering capacity of a recirculating system. Monitoring of water for ammonia or excessive pH decreases should be the basis on which a decision is made whether a system is overcrowded. In addition to the possible degeneration of water quality, crowding may result in immunosuppression. One study (*32*) showed that a pheromone-like substance found in crowded aquaria depressed the immune response of the blue gourami (*Trichogaster trichopterus*) to infectious pancreatic necrosis virus. Collins et al. (*33*), using channel catfish, could not demonstrate that crowding fish at the rate of 171 g/liter of water affected the humoral immune response. Pheromone-induced immunosuppression will likely vary with fish species as well as the conditions under which fish would be immunologically suppressed.

Stress and Infectious Diseases

The concept that infectious diseases in fish are precipitated by a variety of stressors is well-documented (*5, 6*). Also well-established is that various stress situations increase serum cortisol levels in fish (*34–37*). Evidence on the association of glucocorticoid activity with a depressed immune response in mammals is ample (*38–40*). Immunosuppression as a result of hormonal imbalances in fish is assumed, but experimental evidence is lacking.

HEALTH MAINTENANCE OF LABORATORY FISH

Experimentation spanning periods of several weeks to several months frequently involve disease problems which can terminate an experiment prematurely. Inasmuch as the emergence of a disease during an experiment will lead to deaths even with specific treatments, the researcher should attempt to avoid problems before the initiation of the experiment. Therefore, his successful management of an experiment depends on starting with and maintaining healthy fish.

Source of Fish

Fish can be collected from the wild, propagated in ponds, or raised in aquaria. From a disease viewpoint, wild-caught or pond-reared fish have had more exposure to disease-causing organisms than those reared in aquaria supplied with dechlorinated water and in which routine prophylactic treatment is facilitated.

Wild-caught fish frequently are infested with various parasites and have the added disadvantage of being heterogeneous with respect to age and parentage. Pond-reared fish, such as channel catfish (*Ictalurus punctatus*) or Florida-raised varieties, are more likely to be genetically homogeneous and of the same size and similar age group. However, pond-raised fish are also subjected to various parasites, some of which cannot be controlled effectively; for example, metacercariae of digenetic trematodes cannot be physically removed from fish, nor can sporozoan parasites be eliminated. Mycobacterial infections can be a problem in fish reared in ponds.

Fish raised in aquaria in which both genetic factors and disease control can be closely monitored are optimal as experimental animals. In aquarium systems, parasites normally associated with pond rearing can be eliminated, and bacterial problems can be controlled by the avoidance of stress through maintenance of acceptable water quality and use of medications. Even in hatcheries with closed multiaquarium recirculation, tank-to-tank spread of disease can be avoided by exposure of water to UV light (*41*).

Quarantine and Examination

Fish used for experimental purposes should be held in an aquarium system in which water consistent with the good health of the fish is provided (table 1). The quarantine period allows 1) the fish to acclimate to a new environment and 2) the investigator time to examine and treat any disease problem. Quarantined fish held as a group in one large holding vat simplifies feeding and routine treatments and reduces variation in water quality.

Examination of fish should be done by individuals familiar with their disease problems; such individuals can be located through contact with local veterinary diagnostic laboratories, colleges of veterinary medicine, fisheries departments, and private consulting firms. A routine necropsy should include parasitological, bacteriological, and histopathological examinations. Parasites are easily visualized microscopically as are granulomas, which are suggestive of mycobacterial infection. Routine histopathological analysis should be included, so that a normal base can be established for comparison purposes in the event that exposure to a potential carcinogen induces histopathological changes. Additionally, histopathological examination may reveal the presence of the initial stages of sporozoan or mycobacterial infections that may be missed by examination of wet mounts.

An aquarist's decision to administer prophylactic treatments during the quarantine period should be based on results of the examination of at least 5 to 10 fish. Specific medication should be directed to a particular disease organism. A diagnostic approach and treatment regimens of common problems are available (*42*).

The investigator must decide whether to continue with an experiment using a particular group of fish depending on the preexperiment necropsy findings. Problems which are not obvious, such as sporozoan diseases, metacercariae of digenetic trematodes, migrating nematodes, and latent bacterial infections, may be exacerbated by exposure to a potential carcinogen which could be immunosuppressive.

Management of Fish During Experimentation

The two most important factors in maintaining healthy fish during an experiment are good water quality and nutrition. Water quality, as previously mentioned, can be a source of stress if allowed to vary beyond recommended levels. Inattention to water quality may be the largest single cause of deaths in aquarium systems. Before an experiment is initiated, water should be analyzed for naturally occurring or industrial pollutants that could be toxic. If pollutants are present, extensive treatment of water by either one or a combination of procedures may be required; these include flocculation, reverse osmosis, and filtration through activated carbon followed by reconstitution of the water (*29*).

The selection of a life support system must be compatible for the fish and for experimental protocol. In a life support system utilizing any type of recirculation, invertebrate life in filters will gradually accumulate that could interact with a particular test chemical. Also, nitrifying bacteria on filter materials in a recirculating system may be inhibited or killed by the addition of a test material and result in the accumulation of ammonia and nitrites. These problems are avoided in a flow-through system in which fresh water is replaced continually. Flow-through systems have the potential of reducing accumulation of wastes and water chemistry changes associated with waste accumulation. Such systems are optimal for carcinogen testing provided that ample water is available and that provision is made for accurate dosing of the test substance. An important point for all to note is that accumulation of waste products encourages the growth of potentially pathogenic bacteria in a culture system.

Regardless of the type of life support system used, periodic water testing should be a part of the investigator's experimental procedure; testing for pH, ammonia, nitrites, and nitrates is usually adequate. In both single aquarium and recirculating systems, water should be changed regularly. The amount to be changed can be arbitrary, such as 10%, each day or week, or, alternatively, water changes could be adjusted so that the nitrate level does not exceed a set limit, i.e., 5–10 ppm. Regular water changes will dilute the chemical being tested and dosage readjustment must be made.

Nutrition is a major problem for researchers in maintaining the health of small species of fish in aquarium systems. Although nutritional requirements for trout and channel catfish have been determined, requirements for most small species have not been defined. Dupree (*43*) suggested that semichemically defined diets are adequate for small fish species provided that the fish accept the food before essential nutrients are leeched. In a recent study, Blazer (*44*) determined that rainbow trout fed diets deficient in ascorbic acid or vitamin E showed significant decreases in humoral and cell-mediated immune responses. Blazer found that both vitamins can be depleted in feeds during commercial processing and storage periods. Therefore, food commonly available for small species may be deficient in these vitamins depending on processing and storage time, and, if fed for months, may lead to increased susceptibility to potentially pathogenic bacteria present in most aquatic systems. If the nutritional status of the food is not known, supplementation with these vitamins may be indicated. The incorporation of gelatin into a supplemented diet will retard leeching of nutrients into the water.

The feeding of fish three or four times a day with an amount of food they will consume in 3–5 minutes is preferable to a large amount at one feeding. This latter method can lead to excess uneaten food that results in bacterial blooms and an accumulation of ammonia. The technician must remove uneaten food and fish wastes by regular siphoning and during water changes.

Mortality of Fish During Experimentation

An established diagnostic procedure should be followed if fish die during the course of an experiment. Water quality must be checked immediately for temperature, pH, ammonia, nitrites, or other suspected pollutants. After moribund fish are examined for parasites and bacterial infections, a representative number should be fixed in 10% neutral buffered formalin and examined for histopathological changes. Results of a complete necropsy will allow the investigator to determine the course of action. Bath treatments with formaldehyde or other antiparasiticides may be warranted for cases of parasitism of external surfaces (*42*). Hemorrhaging may indicate a systemic bacterial infection which often is precipitated by environmental stress. In screening procedures for carcinogens, not only direct histopathological changes may be associated with various organs but also an immunosuppressive action without gross or histopathological changes. For fish with bacterial infections, specific antibiotic therapy should be based on isolation, identification, and antibiotic sensitivity testing. Because sick fish do not eat, antibiotics are best administered by addition to the water. Guidelines for antibiotic therapy are available (*42*).

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Procedures for Use of Freshwater Fishes in the Development of Reproducible Toxicological Information[1]

Leif L. Marking[2]

ABSTRACT—Toxicity testing with fish began early in this century, but standardized methods have been developed only within the last three decades. Standardized test procedures promote reproducibility of results; healthy fish properly handled and acclimated to test conditions are a given prerequisite. The principles of acute toxicity testing are important in the design of chronic tests for suspected carcinogens because certain factors influence the activity of chemicals or contaminants. The pH of test water is a critical factor in governing the uptake of chemicals by fish. Buffering is required so that uniform pH in waters of different hardnesses and different pHs in water of a given hardness are maintained. The importance of water quality control is graphically demonstrated by the lampricide 3-trifluoromethyl-4-nitrophenol; the toxicant is over 50 times more toxic in water at pH 6.5 than at pH 9.5. Results of laboratory tests on toxicity or carcinogenicity of single compounds in a clean environment represent an oversimplification of the real world because organisms are actually exposed to multiple chemicals or stresses. Because the environment is a complex interaction of physical, chemical, and biological factors that are extremely variable and dynamic, simulation of these systems in the laboratory is, at best, artificial; therefore, results developed must be considered to be predictive. — Natl Cancer Inst Monogr 65: 195–199, 1984.

Toxicity testing with aquatic organisms began in the early part of this century. Doudoroff et al. (*1*) and Hart and associates (*2*) were among the first to recognize the necessity to standardize test procedures. Guidelines were originally developed for investigations of toxic properties of wastewaters; however, they provided important concepts for later toxicity tests with aquatic organisms and for the assessment of the safety of thousands of pesticide formulations to man and the environment. Methods, procedures, and standards for aquatic tests evolved over three decades that followed publication of the original guidelines (*3–9*). Changes in the more recent guidelines reflect registration requirements of the United States Environmental Protection Agency and the Food and Drug Administration.

My purpose here is to summarize some of the methods and procedures used in standardized laboratory tests that lead to reproducible results accepted by the regulatory agencies for acute toxicity testing and to relate their applicability to chronic testing of carcinogens. Our basic methods closely parallel those of the American Society of Testing and Materials and American Public Health Association; however, additional techniques or modifications are required for full assessment of the potential environmental impacts of a chemical. Not all details for aquatic toxicity tests are included because many are readily available in the literature. Emphasis is placed on standardized procedures and special techniques that help one in assessing toxicity of chemicals to aquatic organisms, especially those that may be useful in the chronic exposure of fish to suspected carcinogens.

TERMINOLOGY

In aquatic toxicology, the concentration calculated to produce LC50 is appropriate for describing toxicity because the fish are exposed to concentrations of a substance in the water that surrounds them (*8*). Therefore, they are not receiving a dose (i.e., a lethal dose that will produce 50% mortality) in this kind of exposure. Median tolerance limit has also been used synonymously with LC50, especially in earlier reports, but that term is inappropriate. Tolerance is the ability of an organism to show less response to a specific dose of a chemical than it showed on a prior occasion from the same dose. The term should be used only in that sense (*10*). Also, tolerance should not be used synonymously with resistance.

CARE AND HANDLING OF FISH

Freshwater fish used in tests at the National Fishery Research Laboratory are usually obtained from federal, state, or private hatcheries that have been certified as disease free. Primary species are rainbow trout (*Salmo gairdneri*), fathead minnow (*Pimephales promelas*), channel catfish (*Ictalurus punctatus*), and bluegill (*Lepomis macrochirus*), but additional species are used as required so that use pattern criteria can be developed. The fish are generally young and weigh between 0.5 and 1.5 g. They are fed commercial diets and observed daily for incidence of disease or stress during a 10-day pretest period, under the care of a trained fish culturist (*11*). If fish must be treated for disease, recognized therapeutants are administered (*9*), and the fish are quarantined for an additional 10 days.

Before fish are exposed to chemicals, sufficient numbers for a toxicity test are isolated from the source tank (2,000

ABBREVIATIONS: LC50 = the concentration calculated to produce 50% mortality among exposed organisms; TFM = 3-trifluoromethyl-4-nitrophenol.

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[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

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liters), placed in smaller tanks (50 liters), and fasted for 3 days for minimal accumulation of metabolites and wastes that result when they are placed in exposure vessels (*12*). The eliminated fish are then acclimated to the test water and laboratory conditions for 1 day. To ensure that oxygen concentrations remain at 50% of saturation or more during a 96-hour static exposure, we never allow the loading rate to exceed 1.0 g/liter at 12° C and less at higher temperatures. After the total 4-day acclimation period, the fish are exposed to selected chemicals for 96 hours in static tests and for longer periods in flow-through tests. Information on survival and mortality is recorded at 1, 3, and 6 hours on the first day and daily thereafter. Dead fish are removed daily.

Coldwater species are preferably maintained at 12° C and warmwater species at 17° C. Despite some recommendations that fish be cultured at higher temperatures, these temperatures are adequate to allow some growth and increase the carrying capacity of holding tanks. For tests that assist us to evaluate the effects of temperature, coldwater and warmwater species are acclimated to 7° or 17° C and 12° or 22° C over a 24-hour period, respectively. The temperature change never exceeds 5° C over a 24-hour period. I have found no significant differences in acute toxicity responses of fish acclimated to changes of 1°, 2°, or 5° C per day, but more extensive acclimation periods may be necessary for other research purposes.

PREPARATION OF EXPOSURE WATER

Standard reconstituted water is preferable to other test waters because the components and characteristics can be consistently delineated. Although reconstituted waters can be prepared from almost any source, groundwater is generally the most practical because little or no pretreatment is required. With groundwater, carbon filtration is preferred for removal of organic contaminants, and the water should then be distilled or deionized so that all minerals are removed. The water to be treated should meet the specifications given by the American Society of Testing and Materials Committee E-35 on Pesticides (*9*). Salts added to the demineralized water produce water types that consistently yield the characteristics listed in table 1 (*13*).

The soft water formulation is the primary one used for most routine toxicity tests; its pH ranges from 7.2 to 7.6 and total hardness (as calcium carbonate) from 40 to 48 mg/liter. Temperatures of this water can be altered if an investigator wishes to study the effects of temperature on toxicity, and pHs of this formulation are readily altered by the addition of buffers for determinations of the effects of pH on toxicity (*9*).

The other formulations of reconstituted water listed in table 1 are useful for simulation of other waters. The pHs of these formulations change with differences in total hardness. Therefore, to study the individual effects of water hardness on toxicity, one has to ensure that the pH is maintained at 8.0 to 8.4 by incorporating 384 mg/liter of sodium bicarbonate into each formulation (*14*).

INFLUENCE OF TEMPERATURE, HARDNESS, AND pH ON TOXICITY

The lampricide, TFM, is an effective toxicant against larval sea lampreys (*Petromyzon marinus*) in tributaries of the Great Lakes (*15*). Successful control of this predator has permitted a dramatic comeback of lake trout (*Salvelinus namaycush*) and other native species and the successful introduction of Pacific salmon (*Oncorynchus*).

Several factors responsible for alterations in the toxicity of this lampricide were isolated during the development of information to support registration by the Environmental Protection Agency; temperature, hardness, and pH all influenced toxicity (*16*). The data in table 2 illustrate the importance of water characteristics on the toxicity of TFM to rainbow trout. Like other chemicals, the lampricide tends to be most toxic at high temperatures. The most dramatic influence is exerted by pH; the 24-hour LC50 is 0.699 mg/liter at pH 6.5 and 56.5 mg/liter at pH 9.5.

The accuracy and reproducibility of data such as those presented in table 2 depend on special techniques that one can use to minimize variation. For instance, the trout used for all exposures were from the same source group, of the same age, and were size selected so that grouping of large and small individuals was avoided. Influences of temperature on exposures were investigated simultaneously, those involving water hardness on the following day, and those involving pH on the third day. An even more appropriate procedure would be that one expose trout to all variations on a single day, but few laboratories are equipped to have that capability.

Water is the preferred medium for preparation of stock solutions of chemicals; however, because most organic compounds are not highly soluble in it, acetone or methyl alcohol are generally preferred to dissolve most organic chemicals before they are dispensed into the test vessels. Proper mixing of solubilized chemicals is necessary to ensure uniform concentrations in each vessel and consistency of solubility between vessels. The uptake of chemicals

TABLE 1.—*Quantities of reagent chemicals and characteristics of reconstituted water*

Water type	Salts required, mg/liter				Total as calcium carbonate, mg/liter		pH
	Sodium bicarbonate	Calcium sulfate · water	Magnesium sulfate	Potassium chloride	Hardness	Alkalinity	
Very soft	12	7.5	7.5	0.5	10–13	10–13	6.4–6.8
Soft	48	30	30	2.0	40–48	30–35	7.2–7.6
Hard	192	120	120	8.0	160–180	110–120	7.6–8.0
Very hard	384	240	240	16	280–320	225–245	8.0–8.4

TABLE 2.—*Toxicity of lampricide TFM to fingerling rainbow trout in water of selected temperatures, hardnesses, and pH*

Temperature, degrees C	Water hardness	pH	LC50 and 95% confidence interval, mg/liter at:				
			1 hr	3 hr	6 hr	24 hr	96 hr
7	Soft	7.5	5.17	4.05	2.90	2.64	1.16
			4.20–6.36	3.38–4.87	2.21–3.80	1.97–3.54	0.765–1.76
12	Soft	7.5	5.00	2.93	2.32	1.16	0.710
			4.10–8.10	2.25–3.82	1.82–2.95	0.692–1.94	0.549–0.919
17	Soft	7.5	3.08	3.08	2.89	2.32	1.28
			2.39–3.97	2.39–3.97	2.22–3.76	1.82–2.95	0.975–1.68
12	Very soft	8.2	18.6	14.4	13.4	10.0	5.55
			16.2–21.3	13.0–16.0	11.2–16.1	8.93–11.2	4.42–6.96
″	Soft	8.2	16.4	11.3	10.7	10.7	5.50
			14.5–18.5	9.58–13.3	8.97–12.8	8.38–13.7	4.38–6.90
″	Hard	8.2	21.9	14.6	12.8	10.9	7.90
			17.0–28.2	13.1–16.3	10.8–15.1	9.16–13.0	6.75–9.25
″	Very hard	8.2	21.4	15.6	15.0	12.8	6.75
			19.3–24.0	13.7–17.7	13.4–16.8	11.0–14.9	5.63–8.10
″	Soft	6.5	1.21	0.871	0.750	0.699	0.615
			0.813–1.80	0.704–1.08	0.614–0.915	0.555–0.880	0.400–0.945
″	Soft	8.5	41.1	31.3	28.5	21.3	20.5
			36.9–45.8	27.6–35.4	25.8–31.5	17.8–25.5	16.7–25.1
″	Soft	9.5	—	82.0	73.5	56.5	—
				67.2–100	64.8–83.3	42.3–75.5	

by fish is influenced by ionization factors, and lipid-soluble forms tend to move readily into the fish across the gill membranes (*17*).

The rate of deactivation of antimycin, a registered fish toxicant, has varied widely under different water quality conditions. Earlier, I (*18*) reported that antimycin placed in water at pH 7.5 and 12° C had a half-life of biological activity of 5 days. Additional studies showed that the half-life for antimycin ranged from 310 hours at pH 6.5 to a mere 1.5 hours at pH 10 (*19*). This information demonstrated that antimycin was ineffective at pH 9 and above because of its lack of persistence. The data also demonstrated that antimycin was effective at lower pHs and that it did not persist long enough to interfere with restocking.

The deterioration rate of a chemical is often referred to as its half-life, i.e., the time required for distintegration of one-half of the atoms (*20*). Chemical half-life is a routine determination, especially in studies involving radioisotopes. However, determination of the rate of loss of biological activity of a chemical may yield different results because breakdown products, as well as the remaining parent compound, may contribute activity (toxicity).

ADDITIVE TOXICITY OF CHEMICALS OR STRESSES

Investigators have used standardized laboratory toxicity tests with fish extensively for defining the toxicity of individual chemicals or stresses to fish; most of these data are useful for predicting hazards or for developing chemical registrations. However, in nature, fish and other organisms are often exposed to combinations of toxic substances. These exposures may result in expected (additive toxicity) or unexpected effects (greater than or less than additive toxicity), and any type of additive action could promote adverse effects, such as increased acute or chronic toxicity, inhibition of growth and reproduction, or even tumor production. The mechanisms involved in the toxicity of mixtures are not fully understood, but projected theories include increases in the rate of uptake, formation of toxic metabolites, reduction in excretion rates, alteration of distribution, and inhibition of detoxification mechanisms (*21–24*). Consequently, laboratory data on the toxicity of individual chemicals to fish may represent an oversimplification of the complex actions of combinations of chemicals and stresses within an environment.

Marking and Dawson (*25*) adapted existing methods, concepts, and terminology of additive toxicity to derive a quantitative index for expressing the toxicity of mixtures of chemicals in water. Their additive index concept was useful for evaluating the toxicity of mixtures of chemicals to fish. For instance, the lampricides TFM and Bayer 73 are commonly applied to a 98:2 ratio, and the combination produces additive (expected) effects. Mixtures of malathion and Delnav, two organic phosphate insecticides, have been synergistic against insects, and their toxicity to fish was significantly greater than additive. This kind of information is valuable for our understanding how the total toxic burden of contaminants, including metabolites, may impact organisms in laboratory tests as well as in the environment. Again, the accuracy of this procedure is influenced by techniques, such as exposure of fish to chemicals, individually and in combination, on the same day.

DISCUSSION

In work at the National Fishery Research Laboratory, the staff has attempted standardization of experimental conditions for determining toxicity values, but not all variability will ever be eliminated. The use of selected chemicals and species as reference standards has been investigated as a possible way scientists can parallel the use of white rats in mammalian studies. Because no single species of fish is representative of all fishes and because no single chemical is representative of all classes of compounds, the use and concept of reference standards is of limited value. The physiological quality of test fish and the conditions under which they are maintained are usually more important than the use of standard reference species or chemicals in the generation of reproducible toxicological information.

Toxicity tests with fish should be so designed that a desired objective can be accomplished. Many test results are influenced by standard methods or practices that have become part of governmental guidelines that regulate testing of chemicals. Certainly, standardized tests are required and should be used for development of basic information that is reproducible, but, beyond that point, consideration must be given to the intended use of a chemical or potential impact of a contaminant. Brown (*26*) stated that "the more standardized the test or test animal, the less applicable the information obtained is likely to become.". A problem with standardization is that so much about it is atypical of the natural environment. Because the environment is a complex interaction of physical, chemical, and biological factors that are extremely variable and dynamic, simulation of these systems is at best artificial, and results developed should be considered only in a predictive sense.

The principles of acute toxicity testing are important in the design of chronic tests for suspected carcinogens because certain factors influence the activity of chemicals or contaminants. The characteristics of water govern uptake, elimination, and metabolism of chemicals by fish; the test water must be consistent in quality for the evaluation of toxicity or carcinogenicity. Toxic metabolites are flushed out of flow-through test systems, but they remain in static test systems to interfere with toxicity or carcinogenicity of the compound being tested. Therefore, consideration should be given to removal of metabolites and contaminants in static test systems. Devices could be as simple as a filter in recirculating water, provided that the test chemical remains in solution.

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Nutritional Requirements of Experimental Fish[1]

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ABSTRACT—Satisfactory fulfillment of the nutritional requirements of experimental fish under subchronic and chronic test conditions (such as carcinogenesis studies) is critical to the outcome of a study. Nutritional requirements of many coldwater, warmwater, and marine fishes have been determined in part by the use of semipurified diets. Generally, these diets were fed to small fish in controlled environment tanks with no supplemental food available. The control diets contained semipurified natural ingredients, or, when appropriate, purified (crystalline) nutrients and were formulated to meet the requirements (approximate) of the test species. We fed the fish representative diets to insure that their nutritional needs were met and that no adverse pathology would develop. We then modified these control diets by removing one or more of the purified nutrients to determine the essentiality of a dietary component. Later, other diets were formulated to contain different amounts of the essential nutrients, or one ingredient was substituted for another, so that the quantitative requirements could be decided. Thus we could determine the dietary requirements for protein, fat, carbohydrate, and bulk, and the qualitative and quantitative requirements for the individual amino acids, vitamins, fatty acids, and some minerals. Semipurified diets were later used in toxicity studies for the measurement of the effect of different levels of manufactured contaminants, such as insecticides, or natural toxicants, such as aflatoxin, on the well-being of fishes. Information on the preparation of semipurified diets for fishes, feeding techniques, and nutritional requirements as well as suggestions on care of small fishes in tanks appropriate for longer term carcinogenesis studies is provided. — Natl Cancer Inst Monogr 65: 201–209, 1984.

The use of fish as test animals in physiological and toxicological experiments is increasing. The desirability of fish in these tests are many: The researcher can regulate metabolic rate by controlling water temperature, expose the test animal to various drugs or chemicals by bath, injection, or diet, and can utilize many thousands of siblings in a single study. Most importantly, the researcher can reduce the cost of securing and maintaining test animals. Some fish species, such as the brown bullhead catfish and rainbow trout, can be easily grown and maintained in small test facilities as 1- to 100-g individuals, or, if larger specimens are required, they can be reared to a kilogram or larger size. Fish that can be used in research as test animals may be superior in some aspects to some terrestrial animals now being used.

However, the use of fish in experimentation may be more difficult than that with other traditional terrestrial research animals. Fish live in an aquatic environment and thus special problems are encountered with feed formulations, feeding techniques, and environmental maintenance. These and other unidentified problems are not insurmountable. Physiologists and nutritionists specializing in fish research and some medical researchers have shown that this is a good group of experimental animals that will provide excellent research data. I would like to discuss several aspects of the use of fish as test animals, including nutritional requirements, diets and feeds, and some maintenance techniques.

NUTRITIONAL REQUIREMENTS

Research on the nutritional requirements of fishes, primarily the rainbow trout, basically began in the 1930s. This research, similar to the investigations with humans and domestic animals, was hindered by incomplete knowledge of the dietary nutrients, availability of purified and semipurified ingredients, and the lack of a control diet of known content that would support growth and reproduction. Halver (*1*) provides an excellent overview of this early research in fish nutrition.

Our present knowledge of the nutritional requirements of fishes had its beginning with the semipurified diet formulated and tested by McLaren and her co-workers (*2*) and was followed by a series of experiments starting in 1951 by a group of Oregon State researchers (*3–5*). In 1957, another group of investigators formulated and tested a semipurified diet containing vitamin-free casein as the protein source and with purified carbohydrate, lipid, and bulk (*6*). The vitamins and minerals were added as crystalline compounds. This basic diet (*6*) was fed to yolk-sac rainbow trout fry, which were then reared to maturity and spawned each year for 2 seasons. The fry appeared normal in both years and chemical analyses; histological inspections of the tissues of the broodfish and their fry revealed nothing unusual.

These diets (*2–6*) were later shown to be markedly deficient in the $\omega 3$ fatty acids, and, consequently, many of the nutritional studies on trout and salmon and probably other fish species before and indeed after 1972 were conducted with nutritionally inadequate semipurified diets. Sinnhuber et al. (*7*) reported in 1972 that trout require at least 1% $\omega 3$ fatty acids in their diet to achieve maximum growth. The 2% cod liver oil used by Halver and Coates (*6*) and by DeLong and his co-workers (*8*) contained only approximately 0.5% of the essential $\omega 3$ fatty acids, an amount insufficient for the maximum growth or production of healthy fishes.

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[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Fish Farming Experimental Station, Fish and Wildlife Service, U.S. Department of the Interior, P.O. Box 860, Stuttgart, Arkansas, 72160.

Thus with the earlier work on purified diet formulation and testing and the later findings on ω3 fatty acid requirements, the nutritionist was provided with a feed of known composition and content that met the nutritional requirements for growth and reproduction and one in which the dietary nutrients could be independently controlled. These diets also became the basis for much of the later nutritional research with trout, salmon, catfish, common carp, flat fish, eel, and several other species. Excellent reviews are contained in Halver (*1*), National Research Council Nutrient Requirements of Domestic Animals Bulletins (*9–11*), Cowey and Sargent (*12*), and Dupree (*13*). Ketola (*14*) tabulated the amino acid, fatty acid, vitamin, and mineral requirements for fishes.

Protein

Fish require a liberal intake of nutritionally balanced and digestible protein throughout their entire life. In controlled environmental facilities, the total protein component must be supplied in the feed.

The protein required for maximum growth depends on digestibility of the protein(s) and the content of the amino acids essential for the growth of that species. A number of other factors will affect the digestion, absorption, and utilization of protein(s), including the fishes' state of health, water temperature, and the sparing effects of the other nutrients, which, in turn, affect the protein requirement for maximum growth. Thus no exact values can be provided, and it is up to the researcher to determine the protein requirements for his test species in his facilities through the review of the literature and experimentation. Several references (*1, 9–14*) provide data on gross protein requirements.

Sinnhuber and co-workers (*15*) reported that maximum growth of rainbow trout over an extended 4- to 16-month period was obtained when feeding the Oregon Test Diet that contained 49.5% casein, 8.7% gelatin, and 10% salmon or herring oil. However, if fish protein concentrate was used, the crude protein level could be lowered to 40% for maximum growth.

The standard environmental temperatures for salmon, trout, and channel catfish are 10°, 15°, and 30° C, respectively (*9*). Culture of these fishes outside these standard temperatures affects the requirements and utilization of protein. For example, De Long and co-workers (*8*) found that coldwater chinook fingerlings require 40% protein at 8° C and 55% protein at 15° C.

Inspection of the data reported in table 1 reveals that, for channel catfish, high quality animal proteins, such as whole egg, casein, and fish, are superior to the vegetable proteins and the amino acid-incomplete plant proteins. As would be expected, a mixture of animal or vegetable proteins is better than a single animal or plant protein. Feed utilization efficiency decreased by approximately 50% when the fish were fed at 18° C instead of near the standard temperature of 30° C for catfish.

Amino Acids

Protein supplies the amino acids essential for the maintenance, growth, and reproduction of fishes and all animals. An amino acid test diet, developed by Halver and co-workers (*16*) and modified by other investigators, showed that chinook salmon (*16*), channel catfish (*17*), rainbow trout, (*18*), sockeye salmon (*19*), Japanese and European eel (*20*), red sea bream (*21*), and common carp (*22*) require the same 10 amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) in their diets. One can assume that all fish require the same 10 amino acids in their diets. Quantitative dietary amino acid requirements for channel catfish, carp, chinook salmon, and Japanese eel are listed in table 2.

Some pathology has been reported due to imbalances of amino acids in the diets of some fishes. A methionine

TABLE 1.—*Results of the effects of protein and water temperature on the weight gain and food conversion of fingerling channel catfish*[a]

Food item	Expt I			Expt II		
	Gain			Gain		
	Grams	Percent	Feed conversion	Grams	Percent	Feed conversion
Fish meal	248	165	1.6	129	86	3.1
Zein[b]	13	7	31.6	5	0	0.0
Casein	264	176	1.6	136	91	2.9
Whole egg	303	202	1.4	187	125	2.1
Wheat gluten	85	57	5.0	27	18	14.6
Soybean	138	92	3.0	92	61	4.3
Gelatin[b]	84	56	4.9	45	30	8.8
Blood plasma				95	63	4.2
Whole blood				8	5	49.2
Blood albumin				15	10	26.3
All animal protein mixture	352	235	1.2	203	135	1.9
All plant protein mixture	136	91	3.0	58	39	6.8

[a] In the first experiment, water temperature initially averaged approximately 31° C and decreased to approximately 24° C at the conclusion. In the second experiment, water temperature was a constant 18° C. Total feed offered (dry weight) equaled 411 and 394 g in experiments I and II, respectively. *See* (*13*). Feed conversion = dry feed consumed/fish weight gain.

[b] Proteins were supplemented with tryptophan in second experiment.

TABLE 2.—*Comparative amino acid requirements of several species*[a]

Amino acid	Eel fingerling	Carp fry	Channel catfish	Chinook salmon fingerling
Arginine	3.9(1.7/42)	4.3(1.65/38.5)		6.0(2.4/40)
Histidine	1.9(0.8/42)		1.5(0.37/24)	1.8(0.7/40)
Isoleucine	3.6(1.5/42)	2.6(1.0/38.5)	2.6(0.62/24)	2.2(0.9/41)
Leucine	4.1(1.7/42)	3.9(1.5/38.5)	3.5(0.84/24)	3.9(1.6/41)
Lysine	4.8(2.0/42)		5.1(1.23/24)	5.0(2.0/40)
Methionine[b]	4.5(2.1/42)[c]	3.1(1.2/38.5)	2.3(0.56/24)	4.0(1.6/40)
Phenylalanine[d]			5.0(1.2/24)	5.1(2.1/41)
Threonine	3.6(1.5/42)		2.0(0.53/24)	2.2(0.9/40)
Tryptophan	1.0(0.4/42)		0.5(0.12/24)	0.5(0.2/40)
Valine	3.6(1.5/42)		3.0(0.71/24)	3.2(1.3/40)

[a] Information is modified from (*11*). Values are expressed as percent of protein (*in parentheses*); the numerators are requirements as percent of diet and the denominators are percent total protein in the diet. Data for chinook salmon are from (*23*), carp and eel from (*22, 24*), and channel catfish (*25, 26*).

[b] Cystine is absent.

[c] Value includes methionine plus cystine.

[d] Tyrosine is absent.

deficiency observed by Page and co-workers (*27*) resulted in cataractogenesis, and a tryptophan deficiency resulted in scoliosis in rainbow trout (*18*).

Lipids

Researchers have incorporated some animal fats and plant oils into feed formulations to provide energy and to spare protein. Lipids such as fish oil and to some extent soybean oil are included for the essential $\omega 3$ series fatty acids, but, if fed in excess of the dietary $\omega 3$ requirement, these oils can also be used metabolically for energy. Other oils are deficient or devoid of these fatty acids and can only be used metabolically for energy.

Much of the earlier research on lipid requirements addressed the protein-sparing aspect of fat as an energy source instead of the more expensive protein. For example, Dupree et al. (*28*) reported that 7.0- to 7.5-g fingerling channel catfish fed semipurified diets that contained 25% protein and 5, 8, 12, 15, and 20% bleached menhaden fish oil had maximum weight gain and protein deposition with the 15% lipid diet when it was fed at 30° C. Murray and co-workers (*29*), in a feeding study at 28° C with approximately 1-g channel catfish, reported faster growth with diets that contained 12% lipid and 35% protein than with 5% lipid, or with 5 and 12% lipid and 25% protein. Garling and Wilson (*30*) found the protein-to-energy ratio of 7-g channel catfish reared at 26.7° C to be 88 mg protein/kcal between the energy concentrations of 275 and 341 kcal/100 g.

Larger channel catfish require less protein than do smaller catfish. Page and Andrews (*31*) determined the best conversion with a diet containing 35% protein and 12% lipid for 100-g fish; 500-g fish required only 25% protein and 12% lipid.

Water temperature also affects the energy requirement of channel catfish. Approximately 1-g fish when cultured at 28° C grew better with the diet containing 35% protein and 12% lipid than with diets that contained 25% protein or with those that contained only 5% lipid. In contrast, when these fish were fed the same food at 23° C, the diet with 5% lipid and 25% protein was sufficient for maximum growth, which indicated a lower metabolic requirement (*29*).

Lee and Putnam (*32*) found that calorie intake regulated feed consumption of rainbow trout. In studies of their purified diets with 36, 44, and 53% casein:gelatin levels in combination with fat levels (herring oil) of 8, 16, and 24%, the differences were not significant in weight gain except for those fish fed diets low in both fat and protein. Feed conversion values ranged near one unit of gain for each one unit of dry diet offered. Another experiment with purified diets showing the replacement of fish oil with lard was reported by Yu and co-workers (*33*).

Fatty Acids

The need of linolenic acid ($18{:}3\omega 3$) or longer chain fatty acids in the diets of rainbow trout is well documented. Castell and associates (*34*) demonstrated that 1% $18{:}3\omega 3$ prevented the fatty acid deficiency symptoms of fin erosion, heart myopathy, stress shock, and swollen pale livers. The addition of 5% $18{:}2\omega 6$ did not correct these deficiency signs. Yu et al. (*35*) conducted a 34-month study in which rainbow trout were fed a diet that contained 1% ethyl linolenate plus 5% ethyl laurate. These fingerling trout grew to maturity, were spawned, and the resulting fry grew at normal rates. Several reports further substantiated the requirement of the $\omega 3$ fatty acid for rainbow trout (*36–39*).

No quantitative requirement for $\omega 3$ fatty acids has been established for carp, eel, or channel catfish. However, Watanabe and co-workers (*40, 41*) noted better growth of carp fed cod liver oil than with methyl laurate. Channel catfish fed diets containing menhaden oil have grown more rapidly than catfish fed diets containing beef tallow, safflower oil, or corn oil as the major lipid (*28, 29, 42–45*). Takeuchi and associates (*46*) reported better growth of fingerling eel fed 0.5 or 1.0% supplements of methyl linoleate than with 7% methyl laurate. Thus it appears that $\omega 3$ fatty acids are essential in the diets of these fishes.

Quantitative requirements for the $\omega 3$ fatty acids apparently differ for various species. In numerous studies, the requirement for $\omega 3$ by rainbow trout is near 1% of the diet.

In contrast, coho salmon were observed to require 1 to 2.5% trilinolenin for best growth (*47*). Rainbow trout used 5% trilinolenin with no trilinolein or 3% trilinolenin with 1% trilinolein more efficiently than the higher concentrations of trilinolein (*48*).

The redbelly tilapia, *Tilapia zilli*, appears to be different from the other fish observed in that optimum weight gain is obtained by feeding 1% 18:2ω6 or 20:4ω6 fatty acids (*49*).

Carbohydrates

Carbohydrates in the form of starches are included in commercial fish feeds primarily for their ingredient binding characteristics. Carbohydrate will spare protein in fish diets; Ringrose (*50*) demonstrated this sparing effect in brook trout fed marginal protein levels of 28 or 32%, and protein-to-calorie ratios of 75 mg protein/kcal. Moreover, Nail (*51*) reported that 1 g carbohydrate spared 0.05 g protein in feeds for fingerling channel catfish. Dupree and Sneed (*52*) observed with catfish, as did Ringrose (*50*) with brook trout, a better utilization of carbohydrate in low protein and energy diets. In their studies with channel catfish, Garling and Wilson (*53*) found their species used 2.25 g dextrin to replace 1 g lipid for growth.

Maximum dextrin levels which did not reduce growth were 10, 20, and 30% for yellowtail, red sea bream, and carp, respectively (*54*). Digestible carbohydrate should not exceed 30% for trout (*9*). Dupree (*55*) observed an increase in weight gain when the dextrin content of semipurified diets was increased from 8 to 36%.

Dietary fiber in the form of cellulose is often added to semipurified diets; however, studies on its effect are limited. Fingerling channel catfish gained weight when the fiber content of their dry diet was up to 20% (*52*). Leary and Lovell (*56*), working with practical feeds for channel catfish, detected that cellulose levels as high as 8% did not inhibit growth but 14% dietary cellulose depressed weight gain.

Vitamins

Aquaculturists determined over 40 years ago that vitamins or certain feed additives were required in the diet of cultured fish. These early culturists and hobbyists used fresh organ meats and plant products such as yeast to provide the missing nutrient factors. During the 1950s and later when crystalline vitamins had become available, they conducted studies using semipurified diets to determine the qualitative and quantitative vitamin requirements for many warmwater, coldwater, cool water, and marine species. These values are reported in several publications (*9–11*) and in table 3. Deficiency signs are similar for most fish observed [(*9–11*); table 4].

Minerals

Studies on the mineral requirements of fishes are limited. Fish can meet the requirement for some minerals from the water by absorption across the gills; other minerals can be obtained from the food. Calcium is obtained primarily through gill absorption from the water (*57*). Dietary calcium had no observed effect on the growth of carp (*58*), rainbow trout (*59*) or channel catfish (*60*). However, another major component of fish scale and bone, phosphorus, must be supplied in the diet (*21, 58, 59, 61, 62*). Dietary phosphorus requirements for channel catfish was reported to be 0.42 to 0.47% of the dry diet (*61*). Ketola (*63*) with the Atlantic salmon, Yone (*21*) with the red sea bream, Ogino and Takeda (*58*) with the carp, and Ogino and Takeda (*59*) with the rainbow trout determined phosphorus requirements of 0.7, 0.68, 0.7, and 0.8% of the dry diet, respectively.

TABLE 3.—*Vitamin requirements for fishes*[a]

Vitamin	Warmwater[b]	Coldwater[c]
Fat soluble		
A	5,500 IU	2,500 IU
D	1,000 IU	2,400 IU
E	50 IU	30 IU
K	10	10
Water soluble		
Ascorbic acid	100	100
Thiamin	20	10
Riboflavin	20	20
Pyridoxine	20	10
Pantothenic acid	50	40
Niacin (nicotinic acid)	100	150
Biotin	0.1	1
Folacin (folic acid)	5	5
B_{12}	0.02	0.02
Choline	550	3,000
Myoinositol (inositol)	100	400

[a] Requirements are those recommended for the commonly cultured warmwater and coldwater fishes. Amounts shown are milligrams of vitamin/kilogram dry diet unless shown otherwise. These amounts do not allow for processing or storage losses.

[b] Data are modified from (*10*).

[c] Data are modified from (*11*).

The quantitative requirements of magnesium, involved in bone formation and interrelated to calcium metabolism in fish, were calculated as 0.06 to 0.07% for the rainbow trout (*64*) and 0.04 to 0.05% for carp (*65*). An imbalance of calcium to magnesium is possible. For example, Cowey (*66*) fed 2.7% dietary calcium in combination with 0.004% magnesium and detected nephrocalcinosis in rainbow trout. However, increasing the magnesium level to 0.1% prevented the pathology. Marine species are capable of meeting their dietary requirement from the water (*67*).

Carp and rainbow trout developed deficiency signs of abnormal curvature of the spine when fed diets that contained only 4 mg manganese/kg dry diet. The pathology was not observed when the manganese content of the diet was increased to 12 mg/kg dry feed (*68*).

Zinc deficiencies have been reported in carp (*69*) and rainbow trout (*70*). With the trout, 15 mg zinc/kg dry diet resulted in normal growth and no deficiency signs were noted. With only 1 mg/kg zinc in the diet, these investigators saw poor growth, cataracts, and fin erosion. Ogino and Yang (*69*) reported that carp required 15 mg zinc/kg dry diet for optimum growth.

Signs of hypochromic, microcytic anemia appeared when diets deficient in iron were fed to red sea bream (*21*) and carp (*71*). Diets which prevented these symptoms in the red sea bream and in the carp contained 1.2 g ferric citrate and 0.2 mg ferric sulfate/kg dry diets, respectively.

TABLE 4.—*Signs of nutrient deficiencies*[a]

Nutrient	Deficiency	
	Clinical	Subclinical
Fat-soluble vitamins		
A	Impaired growth, exophthalmos, eye lens displacement; edema, ascites; depigmentation	Corneal thinning and expansion, degeneration of retina
D	Poor growth, tetany of white skeletal muscle	Impaired calcium homeostasis
E	Reduced survival and growth, anemia, ascites	Immature erythrocytes, i.e., poikilocytosis; variable sized erythrocytes, i.e., anisocytosis; erythrocyte fragility and fragmentation; nutritional muscular dystrophy; elevated body water, i.e., exudative diathesis; lipid peroxidation
K	Prolonged blood clotting; anemia	Reduced hematocrit
Water-soluble vitamins		
Thiamin	Poor growth, anorexia, hyperirritability, convulsions, loss of equilibrium	Low transketolase activity in erythrocytes and kidney
Riboflavin	Impaired growth, anorexia	Lens cataract, adhesion of lens and cornea, impaired activity of erythrocyte glutathione reductase
Pyridoxine	Poor growth, anorexia, epileptiform convulsions, hyperirritability, lowered resistance to handling, erratic and spiral swimming, rapid breathing and gasping, flexing of opercles, rapid onset of rigor mortis	Reduced muscle and erythrocyte amino transferases
Pantothenic acid	Poor growth and survival; anorexia; clubbed, exudate-covered gills	Atrophied pancreatic acinar cells, vacuoles, and hyaline bodies in kidney tubules
Niacin	Poor growth and feed conversion, anorexia, skin lesions, anemia	Colon lesions
Biotin	Reduced growth and feed conversion, increased mortality, degeneration of gill lamellae, skin lesions, i.e., blue slime	Reduced liver acetyl CoA carboxylase and pyruvate carboxylase, altered fatty acid synthesis, lipid infiltration of liver, degeneration of pancreatic acinar cells, glycogen storage in kidney tubules
Folic acid	Slow growth, anorexia, poor feed conversion, anemia, pale gills	Large, immature, segmented erythrocytes
B_{12}	Anemia	Small erythrocytes, low hemoglobin
Choline	Impaired growth, poor feed conversion	Fatty livers
Inositol	Anorexia, reduced growth, anemia	Reduced cholinesterase
Ascorbic acid	Anorexia, reduced growth, lordosis, scoliosis, lethargy, hemmorrhagic exophthalmia, ascites, anemia, intramuscular hemorrhage	Reduced concentrations of ascorbic acid in liver and anterior kidney; abnormal histology of support cartilage in eye, gill, and fin; reduced serum thyroid hormone (T3); elevated plasma cholesterol and triglycerides

[a] *See* (*10, 11*).

The need for dietary copper has been observed for the carp (*68*) and channel catfish (*72*). In contrast, Ogino and Yang (*68*) could not demonstrate a difference in growth of rainbow trout when fed as little as 0.7 mg copper/kg dry diet. Murai and co-workers (*72*) suggest that the dietary requirements for fish could be less than that for terrestrial animals because fish can absorb the copper ion from the water.

Selenium added to the diet at the level of 0.1 mg/kg dry feed prevented the development of deficiency signs, whereas no supplementation resulted in reduced glutathione peroxidase activity in Atlantic salmon (*73*). Hilton et al. (*74*) reported maximum plasma glutathione peroxidase activity with dietary selenium levels of 0.11 mg/kg dry diet; a level of 13 mg/kg caused reduced growth and death.

Woodall and LaRoche (*75*) calculated the dietary level of iodine for the chinook salmon at 0.6 mg/kg dry diet.

Because a great similarity of enzyme systems exists between fish and terrestrial animals, one might assume that fish require the same minerals as do terrestrial animals.

However, the dietary requirement could be different for fish and terrestrial animals because many minerals are obtained totally or in part from the water.

PREPARATION OF SEMIPURIFIED DIETS

Semipurified diets can easily be formulated for a wide variety of test fish. The feed must contain the essential dietary nutrients in the required amounts for the test fish and be acceptable to the fish in respect to texture, particle size, taste, and flavor. Examples of two purified diets are given in table 5.

TABLE 5.—*Suitable semipurified diet formulations for small-sized fish in carcinogenicity testing*[a]

Ingredient	Percent of dry diet	
	Rainbow trout	Catfish
Casein	49.5	40
Gelatin	8.7	—
Dextrin	15.6	10
Salmon oil[b]	10	10
Vitamin mix[c]	2	2
Mineral mix[d]	4	4
Cellulose flour (bulk)	8.2	28
Chlorine chloride	1	1
Carboxymethyl cellulose	1	5
Water[e]		

[a] All diet ingredients should be analyzed for polychlorinated biphenyls, antioxidants, pesticides, and stabilizers.

[b] Oils must be further analyzed so that an adequate content of the $\omega 3$ fatty acids is ensured.

[c] For recommended quantities required to satisfy the nutritional requirements, *see* (*10, 11, 15, 77*).

[d] For recommended quantities required to satisfy the nutritional requirements, *see* (*76*).

[e] One can prepare the wet diet by mixing ingredients (without the carboxymethyl cellulose) with 100 parts of warm water (40° C) and thoroughly mixing. Test compounds may be added and thoroughly mixed into the feed. While stirring the wet diet, sprinkle in the carboxymethyl cellulose and mix until the feed ingredients are well bound. Cover the feed and store under refrigeration.

Inasmuch as the purpose of the purified diet is the testing of the effects of various compounds added to the water or feed or given by injection, one must be certain that the dietary ingredients and the environment be free of all natural and man-made contaminants. A number of biochemical companies and laboratories have made available semipurified natural nutrient products. However, these products must be analyzed and free of contaminants that may affect the experiment.

In many experiments and especially in carcinogenicity testing, a high quality diet that will result in optimum growth is desired. From the practical aspect, this involves the use of a high biological-value protein(s) at the optimum level for that size and species of fish and sufficient energy (lipid) for near maximum growth. The other nutrients, such as the essential fatty acids, vitamins, minerals, carbohydrate, and bulk, must be added at levels that meet the nutritional needs of the fish (*10, 11*).

MAINTENANCE AND FEEDING OF FISHES

All aspects of maintenance and feeding of fish will not be discussed [*see* references cited; (*76, 77*)]. I would like to relate some of my experiences in the feeding of semipurified diets and the use of fish in laboratory studies.

Sources of Fish

It is difficult for a reseacher to obtain test fish free of infectious diseases and that have not been challenged with potentially cancer causing drugs and chemicals. Usually, the commercially provided fish must be treated for removal of parasites and disease organisms, and the effects of these chemicals and drugs on the fish are generally unknown.

Another major problem with wild or commercially produced stocks is that these fish often do not respond well when placed in tanks and are fed the bland semipurified diet. I have found it difficult to obtain good nutritional and physiological data from fish brought into the laboratory from ponds or other outdoor facilities.

My experiences and those of my colleagues have been much better when the test fish were brought into the laboratory as eggs or as yolk-sac (prefeeding) fry. This procedure has worked well with many species, such as catfish, sunfish, trout, salmon, and eel.

Water Supply and Conditions

When fry-sized fish are to be cultured in the laboratory, one must have facilities that can be cleaned easily and a contaminant-free water supply. I emphasize that this water must be free of dissolved chlorine, and, if heated, the excess nitrogen gas must be "beaten" out and oxygen "beaten" into the water. Other contaminants, such as gaseous hydrogen sulfide, carbon dioxide, and dissolved (ferrous) iron must also be eliminated. Dissolved oxygen should be maintained at or near saturation in tests of these types.

Best results in relation to growth rate and feed efficiency occur when the fish are maintained at their standard environmental temperatures as described in (*9*). From a practical standpoint, it is easier for one to train the fish to feed; their feed consumption and growth is greatest when the fish are maintained at or near their physiologically preferred water temperatures.

Test Facilities

Fish can be sucessfully reared and tested in a variety of facilities, such as troughs, aquaria, or jars. Some may require a flow-through system, but, in other tests, a static system may be desired.

However, in fish research, the water should be kept free of uneaten feed and feces. Prevention of the buildup of ammonia and other waste metabolites in the test environment will reduce stress and usually retard the invasion of infectious diseases and parasites and metabolite-caused pathology (due to stress).

Feeding the Fish

Food should be offered when fry have absorbed the yolk material and are ready to feed. Many fish species have been started on semipurified diets prepared as flake feed, dry pellets, or moist (wet) diet. The technician or researcher must exercise much tender love and care at this stage and encourage all the fish to eat. Fish that do not feed within several hours after seeking food usually never feed and will soon die.

Trout, salmon, catfish, carp, goldfish, and numerous other species can be started on purified diets similar to those described in table 5. These fish can then be grown to the size required for the experiment. Use of purified feed through the total lives of these fish has advantages, the most obvious of which are that the fish were never challenged with a potential carcinogen and their nutritional needs were met.

If the experiment design will permit and if contaminant-free natural feed materials can be obtained, the fish can be started on organ meats (liver and heart), brine shrimp, and other starter feeds. Should it be necessary, zooplankton, rotifers, and similar fish food organisms can be cultured in tanks in the laboratory and used for feed. If the media materials are free of contaminants, the resulting organisms should be as well. Use of natural feeds is to be discouraged if the fish can be started on purified diets.

Fry should be offered feed 8 to 12 times daily, but after several weeks this frequency can be reduced. Small fish for research purposes should be fed at least twice each day. For many experiments and especially those studies in which rapid growth is desired, the fish should be fed all that they will eat within 5 minutes after the food is offered and be fed as many times daily as practical.

Handling of Fish

Small fish that have been cultured in an indoor research facility can usually be removed from there and handled, weighed, or injected. These fish can usually be returned without mortality or cessation of feeding activity. It is essential that one not stress the fish by changing the body temperature or by having the oxygen level fall below 5 ppm in the transfer containers. Larger fish are more difficult for the researchers or technician to handle and are more subject to stress.

Although these comments on obtaining and culturing small fish are not complete in all aspects, they do reflect the type of questions I am asked most frequently on problems encountered in laboratory studies.

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Serological Alterations in Carcinogen-exposed Teleosts: Procedures for Preparation and Analysis of Samples From Small Fish[1, 2]

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ABSTRACT—To study the effects of environmental carcinogens on the immune system of *Cyprinodon variegatus,* we had to miniaturize or modify standard immunological procedures due to the small size of the fish. Modifications in standard bleeding procedures allowed collection of sufficient serum for most serological procedures. Serum electrophoresis showed considerable variation between exposed and unexposed fish as did qualitative immunoelectrophoresis techniques. We successfully adapted a bacteriophage neutralization procedure for use with the *C. variegatus* system to measure antiviral antibodies. The presence of antibody-forming cells in spleen suspensions from fish immunized with human type O erythrocytes was demonstrated by a modified immune rosette procedure. A capillary tube procedure was developed for separation of leukocytes from erythrocytes in blood from *C. variegatus.* — Natl Cancer Inst Monogr 65: 211–216, 1984.

The teleost, *Cyprinodon variegatus* (sheepshead minnow), is a member of the killifish family, Cyprinodontidae. The *C. variegatus* can be found in large numbers in estuarine waters along the Atlantic and Gulf Coasts of the United States. The fish is highly adaptable and can be kept with little difficulty in a laboratory environment. Mature members vary in length from 5 to 8 cm and can easily be differentiated with respect to sex. We are currently using this teleost as a laboratory model in the study of carcinogenesis in vertebrate species. One aspect of the study is the determination of the effects of carcinogen exposure on the immune response of the fish.

The immune system of fish is known to be complex and involves many substances ranging from specific "acute phase" enzymes, to interferon, to immunoglobulin production (*1*). The primary problem investigators encounter in studying the immune response of *C. variegatus* lies in its size. The immune response to specific antigens has been routinely researched in many larger species of fish, such as brown trout, *Salmo trutta* (*2*), and sockeye salmon, *Oncorhynchus nerka* (*3*), in attempts for increased production of economically important species. In studies of the phylogeny of the immune response, large species of shark have also been used (*4*). In work on the humoral immune response of any organism, serum must be easily obtainable in reasonable volumes; quantity is not a problem in larger fish from which blood may be obtained in milliliter amounts by heart puncture or caudectomy. The blood volume of the largest *C. variegatus,* however, is measured in microliters. Therefore, we have had to miniaturize or modify techniques used to study the immunology of the fish and to develop a bleeding procedure that significantly enhances blood recovery from these animals.

MATERIALS AND METHODS

Maintenance of fish.—Fish were obtained from independent suppliers at several locations along the Mississippi and Florida Gulf Coast and transported to the laboratory in insulated coolers equipped with aeration from a pressurized tank. Fish were maintained in 30-gallon glass tanks supplied with constant aeration and filtration. Salinity of the water was adjusted to 10 ppm with a commercial salt mix (Rila Marine Mix, Teaneck, N.J.). The temperature was maintained at 25° C and a 12-hr light/dark cycle was followed. Only sexually mature fish were used; sex of fish was determined and recorded in any experiments in which measurements were made of samples from individual fish.

Serum collection.—Initially, we obtained the blood by completely severing the caudal fin, making a cut from the dorsal to the ventral side. Modifications to the procedure included the combined use of an anticoagulant followed by an anesthetic and by a modification of the cutting procedure. Fish were first exposed to 1% sodium citrate in water for 10 minutes, then exposed to 0.01% MS-222 (both from Sigma Chemical Co., St. Louis, Mo.) in water for 5 minutes. A cut was made (with a scalpel blade) on the ventral side of the caudal peduncle, but the caudal fin was not removed entirely. Rather, the cut was terminated at the backbone, so that the caudal artery and vein were severed but not the spinal cord. Blood was collected in heparinized

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ABBREVIATIONS: MS-222 = tricaine methanesulfonate; PBS = phosphate-buffered saline; L-15 = Leibowitz's L-15 medium; PFU = plaque-forming units.

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capillary tubes, then centrifuged, and the serum was separated and frozen at −20° C until needed.

Collection of leukocytes.—Separation and collection of leukocytes from whole blood of *C. variegatus* was accomplished in 2 ways. In the simplest of the techniques, blood was collected in heparinized capillary tubes, then centrifuged to sediment the cells. The tubes were then broken just above the cells, and the buffy coat (containing the leukocytes) was removed with a capillary syringe. If desired, leukocytes from several fish were pooled in a capillary tube and again centrifuged for reduced contamination with erythrocytes. In the second technique, 20 μl of collected blood was layered onto 20 μl of Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) in a capillary tube and centrifuged at 400×*g* for 30 minutes. Erythrocytes were sedimented at the bottom of the Ficoll-Paque layer, whereas leukocytes formed a loose layer at the interface between the serum and Ficoll-Paque. We placed cells collected by either method on microscope slides, added Wright's stain, and examined them to determine cell types present. We determined cell viability by exposing cell suspensions to trypan blue and observing dye exclusion by viable cells.

Serum electrophoresis.—Serum was collected from fish exposed to 1 ppm benzidine in aquarium water for 7 weeks and from normal controls. Serum was electrophoresed according to the Corning agarose electrophoresis system, which requires a total serum volume of 0.6 μl/fish, for 30 minutes at a pH of 8.6 with a barbital buffer. Bands were stained by amido-black 10B for 15 minutes and then scanned on a Corning densitometer at 500 nm.

Serum immunoelectrophoresis.—To prepare antisera for use in immunoelectrophoresis studies, we collected and pooled sera from 50 fish and diluted the samples with 1:5 PBS at a pH 7.6. The serum dilution was emulsified in a 1:1 ratio in Freund's complete adjuvant. Rabbits were given im injections of 1 ml of this mixture which contained approximately 5 mg of serum protein. Rabbits were bled 30 days postinjection and the sera assayed for anti-fish serum protein activity. Rabbits were boosted as required by sc doses of fish sera in Freund's incomplete adjuvant. Both the Corning and Hyland immunoelectrophoresis systems were used in these experiments.

Immune rosette formation.—Human type O, Rh+ cells were used as the immunogen. Cells were collected, then washed three times in PBS, pH 7.2, and adjusted to a 20% suspension according to the method of Ingram and Alexander (*5*). Fish were given ip injections of 0.1 ml of the cell suspension. Control fish received only PBS. After 30 days, we collected fish spleens and disrupted them in a conical tube cell homogenizer with a loosely fitting pestle to minimize cell rupture. Cells from the resulting pulp were resuspended in tissue culture medium (L-15; Grand Island Biological Co., Grand Island, N.Y.) and passed through fiber mesh for removal of large masses. The resulting suspension, containing predominantly lymphocytes, was collected in a 0.4-ml microcentrifuge tube. Lymphocytes were gently sedimented by centrifugation at 10×*g* and washed three times with L-15. After the last wash, the cells were resuspended in 0.2 ml of a 5% suspension of human type O, Rh+ cells. This mixture was centrifuged for 3 minutes at 10×*g* and allowed to incubate for 1 hour at room temperature or overnight at 4° C. The cell pellet was then gently resuspended in PBS, and a drop was added to a microscope slide. The smear was allowed to dry, examined microscopically for the presence of rosette-forming cells, and then was fixed with Wright's stain and re-examined.

Bacteriophage neutralization assay.—The semimicro bacteriophage neutralization assay of O'Neill (*6*) was used. The neutralization titer of fish sera is defined as the amount of serum required to produce 50% inactivation of the bacteriophage (*7*). Fish were immunized by ip injection of 0.1 ml of an MS2 phage suspension (containing approximately 10^9 PFU in Freund's complete adjuvant. Serum was collected 42 days after injection. Serial twofold dilutions of *C. variegatus* serum were prepared with 25-μl amounts per dilution in Falcon microtest wells. A preparation of MS2 phage (American Type Culture Collection, Rockville, Md.) was grown by the soft agar overlay method of Eisenstark (*8*), and diluted in R medium for phage lysates (Bacto tryptone, 1.0%; Bacto yeast extract, 0.1%; sodium chloride, 0.8%; calcium chloride, 0.02%; glucose, 4.0%) to contain approximately 150 PFU/25 μl. Equal volumes of dilutions of sera (or nonsera controls) and phage suspensions were mixed and incubated at 25° C for 30 minutes. The dilutions were then added to 0.75-ml aliquots of R medium containing 0.8% agar (Difco Laboratories, Detroit, Mich.) maintained at 40° C; to this was added 0.05 ml-aliquot of an overnight culture of *E. coli* K12. The entire mixture was then overlaid on an R medium agar (1.5%) base in 60-mm plastic petri dishes (Falcon Plastics, Oxnard, Calif.). After an incubation of 6 hours at 37° C, the numbers of PFU/plate were counted and the titer established as the highest serum dilution producing at least 50% reduction in number of PFU.

RESULTS

Serum Collection

A comparison of blood volumes collected from *C. variegatus* by different bleeding techniques is presented in table 1. With fish averaging 5.4±0.6 cm in length, the volume of blood collected averaged 18.5±3.4 μl/fish when the caudal fin was completely severed and no treatment with anticoagulant or anesthetic was used. When fish of similar size (averaging 5.5±0.8 cm) were treated with sodium citrate and MS-222, then bled by the modified cut, the volume of blood collected averaged 33.7±16 μl/fish. The wide variation in blood volume obtained from fish when the partial caudal cut technique was used is attributed to the rate of onset and intensity of the contractile response which shuts off the blood flow in the severed vessels. When the caudal fin is totally severed, the contractile response is rapid and strong, whereas partial severing results in varied degrees of contraction. Thus the modifications introduced to the bleeding procedure have significantly increased (almost doubled) the average blood volume obtainable. Omission of any of the additional steps in the modified procedure resulted in the collection of smaller blood volumes.

TABLE 1.—*Comparison of blood volumes collected by different bleeding procedures*[a]

Bleeding procedure	Average length of fish, cm	Average blood volume collected, μl
No pretreatment, caudal fin totally severed	5.4±0.6	18.5± 3.4
Pretreatment with MS-222 and citrate, caudal fin partially cut	5.5±0.8	33.7±16.0

[a] Data are for 15 fish from each group.

Collection of Leukocytes

Both techniques provided successful separation of leukocytes from whole blood. Collection of cells from the buffy coat resulted in more contamination with erythrocytes than collection by the Ficoll-Paque procedure. Leukocyte preparations collected by either method contained cell types identified as normally present in fish blood, based on the comparison of cells in Wright-stained suspensions with descriptions of other cell types (*9*). A high percentage (80–95%) of cells were viable according to our dye exclusion studies.

The availability of a concentration procedure for *C. variegatus* leukocytes has greatly facilitated morphological characterization of the cell types present. The cells have also been used in T-cell rosetting procedures based on nonspecific attachment of erythrocytes to lymphocytes. Although several species of erythrocytes in addition to sheep erythrocytes have been used, no consistent rosetting patterns have been obtained. The fish leukocyte preparations have finally been placed in tissue culture, providing an in vitro system for studies of the activities of immunologically important circulating cells

Serum Electrophoresis

Serum electrophoresis provided base-line data for serum immunoelectrophoresis as well as for its intrinsic value for comparison of control (unexposed) and carcinogen-exposed fish. The small volume of serum required for each electrophoresis run allowed replicate samples of individual fish sera to be tested. Densitometer scans of duplicate runs showed good reproducibility. The results of densitometer scans of serum from control *C. variegatus* and from fish exposed to 1 ppm benzidine for 7 weeks are illustrated in figure 1. A scan of normal human serum is included as a procedural control and shows a typical pattern. Differences between the serum profiles of the control and benzidine-exposed fish are obvious. The control serum shows 8 peaks, with some components evidently present only in extremely weak concentrations or missing altogether. Sera from different controls showed some variation, but were always similar and produced 7 to 9 peaks in densitometer scans, with most producing 8. On the other hand, sera from benzidine-exposed fish showed wide variations in serum profiles, both in size and number of peaks.

Serum Immunoelectrophoresis

The antisera produced in rabbits given injections of pooled *C. variegatus* sera was used successfully in immunoelectrophoresis experiments with sera from control and benzidine-exposed fish. Primary immunization produced unsatisfactory antisera, and a series of booster injections was necessary. Satisfactory antisera were produced after 2 boosters and have improved in usefulness with subsequent boosters. At least 7 precipitin arcs may be seen, one moving slightly toward the cathode and the others showing no movement or movement toward the anode. Some differences between the sera from a control (unexposed) *C. variegatus* and benzidine-exposed fish are apparent (fig. 2); for example, the cathode-migrating arc marked by the arrow is pronounced in the normal serum, but missing (or faint) in the serum from the benzidine-exposed fish. In general, differences from the norm observed in serum from benzidine-exposed fish paralleled differences seen in agarose electrophoresis studies.

Immune Rosette Formation

Immune rosettes were successfully produced and identified in spleen cell suspensions from *C. variegatus*. Figure 3 shows a typical rosette. Complete "halos" of erythrocytes were frequently observed with no leukocytes evident inside. Careful examination of the preparations led to the conclusion that the mounting or staining procedure often caused the destruction of the rosette-forming cells, which evidently

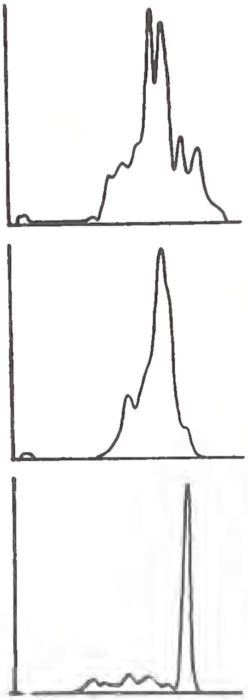
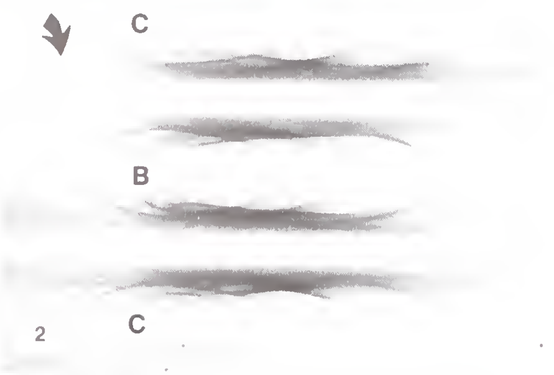


FIGURE 1.—Comparison of serum electrophoresis profiles of normal serum of *C. variegatus* (*top*), serum of *C. variegatus* exposed to 1 ppm benzidine for 7 wk (*middle*), and normal human serum (*bottom*). Fastest migrating peak is at *right* of profiles.



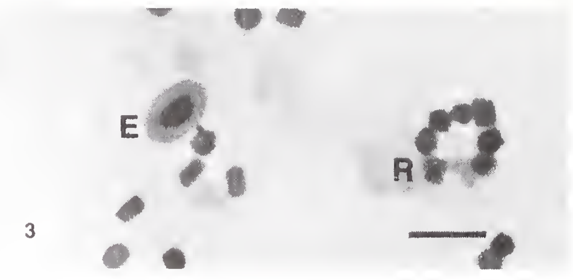


FIGURE 2.—Comparison of immunoelectrophoresis patterns of sera from control (untreated) and benzidine-exposed fish. C = control serum, B = benzidine-exposed serum. Troughs contained rabbit anti-pooled fish serum. *Arrow* points to arc absent in serum from benzidine-exposed fish. Cathode was to *left*, anode to *right*.

FIGURE 3.—Immune rosette-forming cell from Wright-stained spleen suspension of *C. variegatus* immunized with human type O erythrocytes. R = rosette (composed of human O erythrocytes surrounding splenic lymphocyte). E = nucleated fish erythrocyte. Bar = 10 μm.

are fragile. Destruction of such cells within a complete ring of erythrocytes left only the ring with little or no evidence of the rosette-forming cell itself. If only a few erythrocytes were bound to a rosette-forming cell which lysed, it is likely that the rosettes would not have been scored. We had to eliminate this problem for the rosette-forming technique to be of use in quantitating antibody-forming cells in spleen suspensions of *C. variegatus*. Use of a mild fixative prior to mounting of the suspension improved the survival of the cells and the number of clearly indentifiable rosettes, but further improvement is needed.

Bacteriophage Neutralization Assay

Before conducting the bacteriophage neutralization assay with sera from fish immunized with MS2 phage, we did preliminary experiments with sera from control nonimmune fish and from nonimmune fish exposed to 1 ppm benzidine for 7 weeks to determine if any natural neutralizing substances were present (table 2). Sera from controls had no significant effect (significance being determined by reduction of PFU by at least 50%) on the virus titer as may be seen by a comparison of the numbers of PFU in phage inoculum exposed and unexposed to sera. By contrast, significant reduction of PFU was observed if the inoculum was exposed to sera from benzidine-exposed fish at dilutions of 1:8. At a serum dilution of 1:16–1:256 or higher, the plaque reduction was less than 50%, but apparently still present. Further experiments are necessary if we are to demonstrate the dilution necessary to eliminate plaque reduction completely. The results suggest that at least two components of sera from the benzidine-exposed fish reduce plaque titer. One dropped below the minimal concentration for effect at a dilution of 1:16, and the other still exerted an effect at a dilution of at least 1:256. The results further suggest that the plaque reduction is not dose dependent. Additional studies are needed for confirmation of the presence and identification of the serum components.

Table 3 presents the results of an actual neutralization test with sera from immunized and nonimmunized benzidine-treated and untreated fish. Again, the benzidine-treated (unimmunized) fish showed significant antiviral activity (greater than 50% plaque reduction) at a serum dilution of 1:8 and untreated (unimmunized) fish showed essentially no plaque reduction at any dilution. The 50% plaque reduction titer was 1:128 in untreated fish immunized with MS2 phage, and the titer in benzidine-treated immunized fish was 1:256.

DISCUSSION

The role of the immune system of the *C. variegatus*, widely used as an aquatic model in toxicity and carcinogenicity studies (*10–14*) in the response to environmental carcinogens, has been of particular interest to this laboratory, but the small size of the fish presented several obstacles. Whereas extensive methodologies have been developed for scientists to study the immune systems of such common laboratory animals as mice, rats, and rabbits, the smallest of these animals is several times larger than the

TABLE 2.—*Effects of serum from normal and benzidine-exposed* C. variegatus *on the titer of MS2 bacteriophage*

Source of serum	Serum dilution[a] 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	No serum
Benzidine-treated fish	65	65	62	89	113	106	110	117	—
Control fish (untreated)	153	159	132	158	154	152	153	169	—
Phage control (no serum used)	—	—	—	—	—	—	—	—	141

[a] *Numbers represent* No. of PFU. A dilution of phage stock was used which should have produced approximately 150 PFU/inoculum volume.

TABLE 3.—*MS2 bacteriophage neutralization by serum from unexposed and benzidine-exposed* C. variegatus

Source of serum	Serum dilution[a]							
	1:8	1:16	1:32	1:64	1:128	1:256	1:512	No serum
Untreated fish								
MS2-immunized	8	10	27	47	105	165	220	—
Unimmunized	193	201	190	216	202	186	155	—
Benzidine-treated fish								
MS2-immunized	2	3	6	14	25	34	144	—
Unimmunized	70	122	111	116	120	142	221	—
Phage control (no serum used)	—	—	—	—	—	—	—	215

[a] *Numbers represent* No. of PFU. A dilution of phage stock was used which should have produced approximately 200 PFU/inoculum volume.

C. variegatus. Numerous immunological studies involving fish have been made, but most species studied average over 100 g. We found it difficult to obtain sufficient serum or tissue from individual *C. variegatus* to perform standard measures of the immune response, such as precipitation or agglutination assays. The problem was attacked from two directions: We used various methods to improve the volume of serum collected, and, at the same time, attempted to reduce the serum or tissue volume required by miniaturization of appropriate existing procedures or adaptation of procedures that, by their nature, required small sample volumes of the *C. variegatus* model.

The combined use of MS-222, flush exposure to sodium citrate, and a modified incision designed so that a constriction response of the caudal blood vessels was avoided (similar to the autotomic response in lizards) have, it is believed, maximized the blood volume obtainable. The average blood volume collected by this procedure (34 μl) is sufficient for one to perform the immunological assays selected for adaptation to the *C. variegatus* system.

Application of serum electrophoresis to *C. variegatus* serum served to provide base-line data for development of qualitative and quantitative immunoelectrophoresis assays but also showed interesting differences between serum profiles of control and benzidine-exposed fish in preliminary comparison experiments. Hematological parameters of fish have been shown to be sensitive to exposure to environmental pollutants (*15–17*). Complicating interpretation of such studies is the fact that many hematological characteristics vary with season, age, breeding activity, etc. (*17–21*). A complication relating specifically to studies involving serum proteins is the great diversity of these proteins among fish species (*22, 23*) and the fact that the proteins observed do not necessarily have analogs in human or even mammalian serum proteins. These problems emphasize the need for solid base-line research of serum proteins and their variations in any fish species used as a model, and our need to develop culture methods to minimize possible variations caused by seasonal or physiological factors. The application of polyacrylamide gel electrophoresis and isoelectric focusing techniques to *C. variegatus* serum is planned, and the higher resolutions of these methods should clarify any serological protein alterations in carcinogen-exposed fish.

The development of high-titered antisera against pooled *C. variegatus* serum has permitted qualitative comparison of sera by the serum immunoelectrophoresis technique, and the antisera are now being tested in quantitative immunoelectrophoresis procedures (crossed rocket and tandem crossed) that will allow more precise and extensive comparisons. Presently, an antiserum is being developed against purified *C. variegatus* immunoglobulin that will allow simple direct comparison of total immunoglobulin levels by radial immunodiffusion procedures.

The occurrence of immunocompetent cells with the ability to form rosettes has been reported (*24*). These cells occur to the greatest extent in the spleen and to a lesser extent in the anterior kidney (*25*). Rosetting procedures can be used in identification and separation of lymphocyte subpopulations in mammals (*26*), but this is not true with fish because the existence of separate populations of B- and T-cells has to be proved conclusively (*27, 28*). The presence of rosetting lymphocytes from the spleen of an immunized fish can, however, be used as an index of immune function (*24*). Demonstration of immune rosette formation by splenic lymphocytes of *C. variegatus* has been accomplished, although quantitation is at present poor due to fragility of the cells. Modification of standard rosetting methods is in progress and seems to show promise in the evaluation of the immune system of this small fish.

The development of a miniaturized Ficoll-Paque procedure for separation of fish leukocytes is important because it simplifies the study of immunologically active cells in the blood of *C. variegatus.* In a study to be reported shortly, we already have used leukocytes separated from the goldfish (*Carassius auratus*) to determine phagocytic activity of the various cell types present in blood of that animal. This and other types of procedures should be equally adaptable to the *C. variegatus* system.

The phage neutralization test provides us with a simple and sensitive means of detecting antibodies against viral antigen in *C. variegatus.* Serum of nonimmunized benzidine-exposed fish contained substances capable of significantly reducing MS2 phage titer. The substances could be breakdown products of normal serum proteins or substances produced de novo in response to benzidine. Perhaps the benzidine is directly involved. Testing of antiphage activity in separated serum fractions will help identify the substances involved, and such experiments are planned. Before neutralization assays with serum from exposed fish are conducted, one must determine, for each new carcinogen tested, whether such nonspecific phage neutralizers are induced.

In addition to the methods described in this report, development of a competitive enzyme-linked immunosorbent assay for measurement of *C. variegatus* antibodies against a specific protein antigen is near completion. In this assay, *C. variegatus* antiserum against the protein antigen (keyhole limpet hemocyanin) is allowed to compete with enzyme-labeled rabbit antibody against the same antigen for binding sites on the antigen bound to microtiter wells. Unbound antibody is then washed away and a chromogenic substrate for the enzyme is added. If the reagents are properly balanced, the higher the level of antibody in the *C. variegatus* serum, the less enzyme-labeled antibody will bind (due to competition). The amount of color which develops after addition of the substrate is thus inversely proportional to the amount of sample antibody. The competitive enzyme-linked immunosorbent assay will provide us with a rapid, sensitive method for comparing relative amounts of antibody in individual fish sera, requiring approximately 1 μl serum per test.

Although the *C. variegatus* model has presented numerous difficulties for use in immunological studies, methodologies are near completion to allow detailed study of the humoral response and some aspects of the cellular response in carcinogen-exposed fish. The methodologies should also be applicable to other fish species of similar size.

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Panel Discussion[1]

John B. Gratzek[2]

J. Gratzek: How should the effluent from fish carcinogen tests be safely disposed?

A. Guarino: Procedures are already established for disposal of carcinogens resulting from animal experiments from the other programs, and, except for the volumes that you might generate in aquatic experiments, I would imagine the procedures would be the same.

In facing this problem myself, one device which we used to cut down on the volume of disposal was to run the material through a charcoal bed. We had the convenience of a radiolabeled compound available, so we could analyze the effluent. It would be a little more difficult to do it, but conceptually the same thing could be done.

Gratzek: Does anybody have any further comments on that? Do you think carbon would remove just about anything you would want it to remove? Any other suggestions?

W. P. Schoor: At around one-tenth part per billion, charcoal is ineffective for the removal of benzo[*a*]pyrene.

Gratzek: Does anyone have something else to suggest?

Unidentified participant: Charcoal will not remove something like diethylnitrosamine. It is too soluble in water, and therefore is ineffective.

R. O. Sinnhuber: I think one of the best ways to detoxify any substance is to make sure the material goes through the animal before it goes into the water. We do this with aflatoxin B_1 as potent as it is. All the food, at least, goes through the animal first, and the residue is minimal.

The other point to consider is the dilution factor because we are using a flow-through system. The problem is enormous, but we believe we have adequately satisfied the National Cancer Institute that we are not causing a problem.

G. Streisinger: Another possibility which we used is the Ames test. First of all, we asked if our mutagen is present in the concentrations we believe it is present, or is it being absorbed and decreased to a lower concentration by the treatment? That is one aspect that is important, I think. Also, it is an easy test to do. The Ames test tells you if it is there. The next question to ask is "Have we gotten rid of it, when we are all done?"

Guarino: That is an excellent idea.

I. Kimura: When we used the 7,12-dimethylbenz[*a*]anthracene for marine fishes, we boiled the seawater and I burned the remaining salt at several thousand degrees C and destroyed it. When I used *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, we put sodium dioxide into that water, and again it is a satisfactory treatment.

J. J. Stegeman: I would like to ask a related question of people who use these chemicals in water systems. Is there some common procedure for preventing aerosolization of these compounds in systems that foam and when we have air going through the system? What do people do to prevent that problem or to avoid exposure under those conditions?

Guarino: I was referring in my general answer to guidelines that already exist, and I do not know how familiar you are with these, but there are manuals at the National Cancer Institute, if not at the Department of Health and Human Services level, that explain these things.

In regard to volatilization, they recommend that you work in a hood, and they have different classes of hoods. I know that to have an aquarium in a hood is inconvenient. I did not give any details in my answer, like boiling, because the procedures for the use of permanganate, chromate, and combustion, etc. are described in these manuals. Various methods are available.

K. Hoover: In our system, we use the Instant Ocean technique. We had large glass plates custom-built for us that fit right on top of the aquarium, so as to prevent aerosolization of the *N*-hydroxy-2-fluorenylacetamide with which we were working. We also had the technician wear a gas mask when he went into the aquarium room.

Gratzek: Thank you. Dr. Peter Shoor?

Shoor: I want to comment on Dr. Stegeman's question about volatilization. We did an experiment trying to determine the solubility of benzo[*a*]pyrene by following it in water. You can determine that by looking at the volatilization, and the two curves will be the same. We found that chrysene would co-distill at just below the level of solubility, and, if I remember correctly (if you have an average room temperature with average humidity), I think it came to be about 5 pg/cm^3 in a 10-gallon aquarium. I have forgotten what the exposed surface was.

It is definitely a danger, and the lid should be put on it. You do not have to put it in a hood, but I think you should put a lid on it, because they (especially the polynuclear compounds) will co-distill at just below their solubility.

J. A. Couch: Well, the 2 previous speakers have stated some of what I was going to say. However, in the design of our wet laboratory for exposure of fish to various potential carcinogens or concentrations of carcinogens, we looked into the design of glove boxes that will hold aquaria within them. In turn, you can have essentially an aquarium with a lid, as Dr. Hoover mentioned, inside the glove box, to double the protection for personnel and to prevent an aerosol from escaping. We find that in working with the Environmental Protection Agency we must do that for personnel safety whether we like it or not.

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F. Anders: I would also like to comment on the safety of the people, which is most important, in my opinion. For this purpose, we constructed a box and provide gloves for the people who work within the box. Although this is expensive, the people are happier and they can work much better with the substances. It is extremely dangerous, I can tell you. Some of these substances have a short half-life. For instance, *N*-methylnitrosourea has about 1 hour or 1½ hours. It is dangerous to work with. Therefore, I believe that everybody who is working with carcinogens should use or should construct such safety boxes.

Gratzek: My next question is directed to Dr. Hinton. How can investigators design a laboratory study to identify the relative contribution of various components to carcinogenicity observed in a multicomponent natural environment?

D. E. Hinton: With difficulty. In trying to answer a question like this, now, I have to make a couple of assumptions. I assume that a tumor has been found, or a larger number of fish in a small area has been found with a particular tumor. Now the question is how to identify some of the relative contributions of the various components to this cluster that had been seen in the environment.

Well, we heard many of these same kinds of factors mentioned this afternoon in Dr. Martin's presentation. I would divide the study according to environmental factors, which I think he covered in some detail, such as temperature, pH, possible salinity, hardness, and light conditions.

If you want to look at host factors, then you need to know something about the sex of the fish showing the tumor, something about their age, and something about the dietary conditions. We know that, from a nutritional standpoint, if the host has less than optimal protein, it may not respond biochemically when presented with these carcinogenic compounds. So the host factors are important, as is the degree of stress or crowding of the individuals during the time of exposure.

It is extremely difficult for one to try to mimic or even to begin to understand the kinds of things that might be occurring in a multicomponent natural environment. I think, as we heard this afternoon, that we are going to have to begin to consider, and maybe in some complex detail, matrix-type designed studies of one or more of these types of factors.

With the shorter induction systems, we may begin now to look at the effects that the condition of the host may have upon tumor expression, i.e., some of the effects that we could alter in our environment in the laboratory that might affect tumor expression. There is no quick answer to this, as you all know, but I think it is imperative that we begin to look toward more complicated kinds of designs that might begin to help us approach these problems.

R. A. Sonstegard: I think the question is our looking at etiologic factors and epizootic situations of a wild animal population exposed to hundreds, if not thousands, or multiple-log exposures of chemicals. One route that one can use to resolve some of the preliminary considerations, and we have done this type of thing, is called a relay study. You take animal populations that do exhibit a pathobiological response, which you hypothesize has an environmental cause, versus the control population. By supplementing diets and monitoring for associated pathobiological responses, you hope to get the same pathobiological response that was seen in nature, then you can subfractionate and look at the results. You can then look at the chemicals added to mixtures and try to identify them by classes or groups, or perhaps ultimately individual chemicals that could be responsible.

D. Malin: I would like to comment on this issue of the relationship between complex chemical profiles in the marine environment and diseases in organisms.

In Puget Sound, using gas–liquid mass spectral techniques, we routinely analyze some 500 components, and it is obvious that, incidentally, these are conveniently volatile and thermally stable compounds, many of which are known mutagens and carcinogens. If you were to look at polar components, you could probably easily see 1,000 or maybe 2,000 components.

So the question then remains as to how one deals with an environment of this sort when one considers the definite likelihood of antagonism and synergism among these kinds of compounds. I think the approach of taking these compounds out of the environment, such as extracting sediments, has some merit, but like all of the approaches one has, there are limitations.

One worries in extraction techniques, such as using methanol, that one is creating potential mutagens and carcinogens through free radical attacks with oxygen. If one makes up model systems, which is another alternative, one has the problem that the model systems have to be simple, of necessity, and are based on the rather myopic perception which you have through gas–liquid chromatography.

Those sorts of problems clearly exist, and I think it is difficult at the moment to come up with a system which is really going to be entirely satisfactory because, at least as I see it, each one that we have examined has certain inherent limitations. I think we do not understand in any meaningful way, as one of the other speakers correctly indicated, how potent interactions between chemicals are, and, although we may be able to deal with binary and trinary systems in the laboratory at the moment, we have no capability whatever (and I do not really see one for the future) to be dealing with hundreds or thousands of chemicals that exist in the marine environment. It is a complex problem that requires a great deal of thought and effort.

Gratzek: The third question is directed to Dr. Stegeman: In evaluating fish species for carcinogenicity testing, what physiological value should a test fish have for enabling carcinogen test results to be extrapolated to humans?

Stegeman: I see everyone had the same reaction that I had upon first hearing that question. Well, I think that question cannot be answered as stated, and I question its validity. Perhaps, if we address the question of interactions between the chemical and the organism and how physiological variables might influence this, then perhaps it is tractable.

First and foremost, I think, is the pharmacokinetics of a compound, and in these toxicological studies, the matters of uptake, distribution, metabolism, and elimination are part of the paradigm of toxicology that everybody knows is essential.

Given that there is a target organ or target cell a compound is able to reach, then is that organ or cell capable of metabolizing promutagens or procarcinogens? If it is capable of metabolizing these, is it forming the metabolites that are most reactive and mutagenic? If it is forming these, are the adducts with DNA those which are likely to be effective in somehow initiating this transformation? If these adducts are formed, is the repair process efficient enough to eliminate them? If the repair process is not, is the immune system capable of dealing with whatever is left?

So I guess that these factors that we have all been addressing here in this meeting and will continue to address are all related to this question to which we have no simple answer.

If you consider the physiological variables of an animal, such as have been addressed earlier, e.g., dietary considerations, the influence of altered amounts of protein in the diet on the function of cytochrome P-450, the influence of infection on P-450 systems (it is known that in mammals viral infection can depress the levels of P-450), is this specific to certain isozymes of P-450, thereby altering one pathway or another of metabolism of the compound? These are large questions and hard to address. What I would propose is to offer another question for discussion here and that relates to what Dr. Kimura said about nifurpirinol.

As nifurpirinol was found to be carcinogenic in a wild population of fish, which is, presumably, genetically heterozygous rather than homozygous (and Dr. Dawe made a point of this), it was most important that this compound be found carcinogenic in this feral population of fish.

I would like to hear a discussion on the relative merits of using homozygous strains as opposed to heterozygous or feral strains of fish because I think that, within the genetic heterogeneity or uniformity, there are answers to the questions of what physiological variables you seek in a fish as a test organism.

G. S. Bailey: You raised the question of genetic variability versus the possible virtue of using inbred or homozygous strains. That is an extremely important question. No one has pointed out that the use of increasingly inbred lines is a double-edged sword. You may have some advantages from their use.

One advantage is supposed to be, but it has not been demonstrated yet by anyone in this meeting, that you should be able to decrease the variability between experiments for a given dose, and this should allow you, in principle, to use smaller population sizes to obtain significance with your results. We believe that this is an advantage; however, I have not seen any data that demonstrate this.

Another possible advantage would be that if one begins with a heterogeneous population and, by using Dr. Streisinger's technique, for example, creates clones which are homogeneous, one may, by searching those clones, find populations which do differ in varying rate-limiting steps in the carcinogenic process. By identifying those steps and pinpointing those at which differences occur, we may be able to gain some appreciation of the importance of each of those steps as rate-limiting in the carcinogenic process. That is the second advantage of using inbred lines. Areas of variability are identified for one to look at in the absence of any other variability.

From the point of view of using a system which one is trying to create for screening a wide range of compounds, the use of inbred lines would appear to me to be a disaster because you will eliminate the variability one needs to see a response to a broad range of carcinogens. Also from that point of view, in our trout system and I am sure in any of the other systems that people are using, we believe that an inbred line, contrary to earlier suggestions, is not deliberately outcrossed to maintain (and we have checked this) the heterogeneity of the strain that I think is necessary for a screening and mechanism program to maintain this kind of breadth of response.

Couch: I want to comment along the same line. With the exception of the cloned fish, I reiterate, we are not dealing with homozygous fish. Basically, almost all our test fish are heterozygous. Some of them have been inbred, perhaps, or can be inbred, like Dr. Anders' system and others, but basically we are dealing with heterozygous fish, wild fish, brought into the laboratory and held for different periods.

I think the original question was, indeed, a difficult one and impossible to answer, but it could have been more difficult, if phrased differently. You could have been asked to make the extrapolation from neoplasms and invertebrates to man.

Unidentified participant: I do not see that that is any tougher.

Gratzek: This next question will be addressed to Dr. Klaunig. What are the advantages of conducting fish carcinogenicity tests in vitro? Are the results comparable to data from in vivo tests?

J. E. Klaunig: I have listed four advantages which I believe are applicable to most in vitro tests, and these include ease of handling, economy, the ability to make interspecies comparisons, and, finally, the reduced time between the initiation of studies and final data accumulation.

If I could just go back quickly to each of these points. Ease of handling: Cells or cellular extracts, such as S9 or homogenates, can many times be prepared in large quantities and be isolated, and many experiments can be more easily run than the in vivo tests.

Economically, the money spent for long-term bioassays is considerable. As an example, some of the latest quotes for a rodent study are a quarter of a million dollars per compound.

Unidentified participant: That amount is one-half million.

Klaunig: One-half a million? I am using old dollars.

This may not be applicable in the fish because, as we have seen in some of these small fish species, such as the medaka, guppies, etc., they respond fairly rapidly to some of these carcinogenic compounds, and they require fairly low-cost maintenance. You can keep many fish in the space in which you can keep 1 or 2 rats or 5 or 6 mice. As far as economy goes, the short-term tests may not be as applicable, and the short-term in vitro tests may not be more economical than the long-term, with regard to the small fish species.

I think one area in which these in vitro tests have a lot of application is in the ability to make interspecies comparison. By "interspecies," I am not only referring to comparisons between vertebrates, such as man, mouse, rat, bird, and fish, but also to comparisons between different fish.

As I brought out with the liver cell studies, I think that it might be worthwhile, before testing a compound, to isolate cells, such as the liver, from a particular fish or from several fish, and look at characteristics (if you know which compound you want to test), such as carcinogen uptake, metabolism, the ability to bind, or including in metabolism both the activation of the compound and deactivation, the ability to bind to DNA if it is a genotoxic compound, and the ability to either elicit or inhibit DNA repair. If you are talking about cell lines, pure cell lines, you may consider the ability to induce cell transformation. This could be most worthwhile as far as predicting which fish to test with which compound.

Everyone wants to reduce the time between initiation of studies and final data accumulation. Most of the in vitro tests require less than a month, although some, such as cell transformations, go on and on and on. For example, the Ames test is a fairly short test, and the data can be accumulated and used fairly rapidly.

As far as comparing the results from in vitro tests with those of the in vivo situation, I think this is a problem area that needs considerably more work. I think Dr. Bailey will be discussing adducts, adduct formation, and adduct disappearance in vivo and in isolated liver cells. A considerable amount of work needs to be done in this area, not only with fish, but I think even in the rodent and the human systems.

Gratzek: The next question is to be directed to Professor Anders. What preventive measures should be taken to establish a disease-free population of test fish, and what measures should be taken if a disease occurs?

Anders: This is also a question which is difficult to answer. First of all, one has to love the fish. You get up with them early in the morning, give them their food, and go to bed with them in the evening. You also go to them on Sunday, and if all this is right, then the fish will breed well.

In addition, one has to consider some particular matters. Be cautious of the cleaning women. If they clean the tanks, they do it too seriously. The fish do not like clean water. Do not change the water too often. In one abstract I read, the author suggested a 20% change a week; this is too much. Only a little bit of the water needs to be changed.

Another important factor is that the young ones, when they are born, need food immediately. That means there must be some protozoans and such animals in the tank.

J. Schultz: I have heard the same comment concerning excessive water changes from Dr. Kallman who works with the same fish. Something in platyfish may require this old, seasoned water, but I find that it is not harmful and, in fact, seems desirable to change the water. One need not change the total tank but to delete some of the water from time to time, and perhaps 20 or 25% is not bad.

The issue you make about cleaning the tanks excessively is a problem we face continuously in our laboratory. The people (technicians) like a nice, clean tank. As you suggest, I would emphasize again that when new fish are born, if you are not there to feed them immediately it is desirable that they have some feed to browse on, or they do not seem to do well ever again, no matter how well you treat them.

Anders: It might be that I have not expressed myself correctly. In our circulation apparatus (we have a continuous flow-through system), the water becomes changed in total in about 3 weeks. Change is necessary.

Hoover: Mycobacteria is a prevalent problem in aquarium fish. I would like to ask you, Dr. Gratzek, if there is a way that you can effectively eliminate this from experimental colonies. How do you treat the tank to make sure that the problem is not going to recur?

Gratzek: In treating a contaminated tank, you must absolutely sterilize it. The many species of mycobacteria are all around us, e.g., *M. marinum* and *M. fortuitum.* The method used also depends on what fish you are talking about. Which fish are you talking about?

Hoover: The guppy.

Gratzek: If you were to try to establish a mycobacterium-free fish stock, I definitely would get a stock of fish that was raised in an aquarium, not in a pond. These mycobacteria are all around us, really, and so you have to start with a good stock.

Watch your food. I am leery of some of the livers, especially bovine livers, and similar feed. If you have a contaminated tank in the aquarium, I think you must sterilize it.

Hoover: How do you do that sterilizing?

Gratzek: With chlorine.

M. Sigel: Dr. Gratzek, you and I attended a meeting on immunization of fish many years ago in Nebraska, and I imagine that some progress has been made in developing vaccines for various fish pathogens, although I have not been following the topic closely. In response to Dr. Anders' question, if one knows the real pathogen in question, I assume one could attempt immunization.

Unfortunately, I said "attempt." It may not be successful. Other methods have to be considered including antibiotic treatment for bacteria, and perhaps a kind of approach we are using, with *Ecteinascidia turbanata,* which is an organism that promotes activation of the nonspecific immunity against many pathogens. We do not have enough solid evidence for it, yet; although it has worked so far, this approach needs to be substantiated. These are points to be considered.

Many of you do not have diseases among your fish, and you really are fortunate. You have healthy specimens without disease, so that maybe immunization against pathogens is strictly theoretical for you because the fish are reared in the laboratory or because they are genetically homogeneous; I do not completely understand that. I am, as a microbiologist, a little bit surprised that one can really work in the open systems that subject the hosts to stresses and strains of temperature and chemicals, etc., and still not sustain infection and disease in the test animals.

As I say, you are lucky, but the luck may not hold up forever, in which case we had better do more to find out about the causes of bacterial and viral infections and learn more about how to cope with them, besides learning how to wash the tank. I think washing is not going to solve our infection problems, not for a long time.

Stegeman: May I make a comment about coping with bacterial and viral infections? If they are bacterial, the use of antibiotics could provide a complicating factor that might be difficult to handle.

A recent paper described the fact that erythromycin (I think) is processed by cytochrome P-450 and is a suicide substrate, so that an antibiotic can affect the capacity of the system that is activating or metabolizing a compound in which you are interested.

I might also comment that, in a discussion I was having with Dr. Bend, he suggested that research horizontally is possibly not conducive to progress. If you consider all the data we have seen on the slides today and if that had all been done with one species, where would we be?

We just do not know enough about any single species to be able to select them, I guess. Maybe that is a reflection of the fact that we do not know what physiological values of a fish to examine.

J. D. Hendricks: What Dr. Anders has said concerning conditions for rearing aquarium fish are certainly not suitable for salmonids or other large fish species. With these fish, it is highly desirable to have a flow-through system with a high rate of water exchange, frequent cleaning of the tanks, and care of equipment. There is a great difference here in species and in how you handle them.

H. Dupree: As far as all the nutrition and other studies which I have done, in which I would take fish out of the field, i.e., from our ponds which are maintained well, they were never anything but trouble. We started all the investigations with eggs and maintained them in the laboratory, all the way up to the size required for the study. Whenever I took fish from the field, I had bacterial problems, disease problems, and many nutritional problems. Once I stressed the fish with whatever test that I had, they would die before I was able to confirm what I wanted to do.

I believe that we have to spend the money to go ahead and build the facilities to raise good, clean fish either in our laboratories or buy them from a commercial supplier. Do not buy them from Arkansas, Texas, or Pennsylvania or from a supplier who gets them out of a pond and ships them to you in a plastic bag. Suppliers in Florida do the same thing.

Streisinger: I do not want to beat a dead horse, but with regard to flow rate, Dr. Anders, we replaced 20% of the water a day through our constant flow system. I think a lot depends on the density of the fish. If you have few fish per tank, perhaps it is not so important to replace the water.

My other comment relates to this last discussion on how to cure diseases. Our experience has been that when fish were obtained from wholesalers or pet stores, the fish were almost always diseased. The only time we get fish from a commercial supplier is when we read about the color mutant or some other factor and we want to look at it.

We find that atabrine treatment with a slightly raised salt concentration for about a week is moderately effective with a number of diseases. Perhaps someone could give the proportions again. We have really tried a great many ways to clean up zebra fish. That treatment was one of the few ways that did not kill but helped them.

Debate: Mouse Versus Minnow: The Future of Fish in Carcinogenicity Testing[1]

Clyde J. Dawe[2] *and* John A. Couch[3]

I. THE DEVIL'S ADVOCATE'S SIDE: *CLYDE J. DAWE*

We who are participating in this Workshop may be likened to those in church on the Sabbath. We already believe. We have in our pockets well-documented evidence that small fish species develop neoplasms in response to certain compounds. We are persuaded that small fish *could* be used in carcinogenicity testing. Now I am going to switch character.

As Devil's Advocate, a role in which I must delight, since I accepted it, I am here to tell you something you should already know: That no sooner than you say "Amen" and leave this sanctuary, you will be in the secular world of competitive technology, peer-review funding judgments, and hard-nosed administrators, not to mention a lay public that, whether you like it or not, ultimately holds the purse strings. A considerable part of this lay public takes a dim view of evolutionary theory and the concept of the basic unity of life forms. I will not mention any names.

Before I go further, let me introduce my client: Mr. Mephistopheles Mouse, often known by the nickname of Mickey. Oddly, his female consort goes by the name of Minnie, which was the name used for any kind of small fish, usually used for bait, when I was a kid. Together (fig. 1), Mickey and Minnie are quite prolific, and at 2 months of age start producing litters of 6–10 pups every 21 days. Although their life-span is only 36 months in the extreme, by the time they end their reproductive days at around 9 months, it is no problem for them to have several thousand living descendants. As a result of some kinky sex habits encouraged by scientists, hundreds of different strains of homozygous mice now exist and hundreds of mouse genes have been mapped to their appropriate locations on 20 chromosome pairs.

Mickey and his mistress now enjoy a wide supporting cast, but in the early days of the NCI, there was a great deal of scientific skepticism about the relevancy of mouse cancer to human cancer, so much so that NCI was scathingly referred to as the "National Mouse Cancer Institute" (*1*). It is hard to say whether it was cancer research or Walt Disney's imagination that did most toward the anthropomorphizing and acceptance of my client. The point of this facetiousness is: You are dealing with an adversary who is firmly entrenched in the thinking of the general public. If it was hard to convince John Q. Public that carcinogenesis in mice may be something like carcinogenesis in man, how hard will it be to do the same for fish?

Let us turn to the scientific public. The scientific choice of a surrogate for man would be an animal that is exactly like man in every respect but goes by some other name. Fortunately for *all* ethnic groups, as I hope everyone here will understand, this idea has not been accepted any more than the idea that the works of Shakespeare were written by a man who lived at the same time as Shakespeare and was just like Shakespeare, but was really somebody else.

Next in line would be some nonhuman primate. Remember that the present (December 1981) Acting Director of the Division of Cancer Cause and Prevention built his career largely around the use of primates for carcinogen testing. However, nonhuman primates are getting too scarce and too expensive. So where do we turn? Or, put another way, how does one select an animal bioassay system with which a savvy science administrator is bound to fall in love? Let me count the ways he loves thee, bioassay (table 1).

First take the matter of sensitivity. Everybody loves a more sensitive assay system. In rats (close relatives of my client), liver tumors can be induced with aflatoxin B_1 in the diet at a level of 1 part per 100 billion (*2*). Can the rainbow trout or the medaka match this? What data can be produced to show that molecule for molecule of carcinogen, fish (or more particularly, some species of small aquarium fish) are more sensitive than rodents to specific compounds?

On point 2 (a) is it really relevant to put a fish in water containing a carcinogen and, if the fish develops cancer, to conclude that the same compound at the same or some other concentration in drinking water or food would be carcinogenic for man? Rodent bioassay methods have come under criticism for years because feeding or injecting a compound that people are exposed to by topical application or inhalation is said to be unrealistic. For compounds that are absorbed by and are carcinogenic in gill epithelium, one might reason that people with branchial cysts

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ABBREVIATIONS: NCI = National Cancer Institute; IARC = International Agency for Research on Cancer; BP = benzo[*a*]pyrene.

[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

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FIGURE 1.—Mickey and Minnie Mouse.

would be at a high risk. I am reminded here of a little ditty composed by Dr. Malcolm B. Dockerty, one of my preceptors when I was training in surgical pathology:

> "In days of yore we swam about,
> And breathed through gills, just like a trout;
> But when today these gills persist,
> The surgeon sees a branchial cyst."

Luckily, branchial cysts occur only rarely in people, and cancers within branchial cysts even more rarely. Nevertheless, from a scientific point of view, it would be extremely interesting to know whether gill carcinogens are also active in mammalian branchial/pharyngeal pouch derivatives, such as thyroid and parathyroid glands, thymus, and some salivary glands. At present, no compound with carcinogenic activity for gill seems to have been identified.

On point 2 (b) my client has an established record. Quoting from Squire (*3*), "It can be argued that tissue specificity is not always correlated between man and test animals. No biological rules are absolute. However, according to the IARC documents to date, there is an 80 per cent correlation between tissue site susceptibilities in humans and in test animals [mice and rats] among the 15 known carcinogens that have been adequately tested in animals by routes comparable to those of human exposure." An 80% correlation (*3*) is not perfect, but can Minnie Fish beat it? In fact, the only organ in which a good organ correlation seems to be established in fish systems is the liver.

TABLE 1.—*How do I love thee, bioassay? Let me count the ways*

1) More sensitive
2) More relevant to:
    a) route of administration
    b) target organ and cell
    c) the response in man
3) More reliable (fewer false positives and negatives)
4) Less expensive, less complicated
5) Quicker
6) Supplemental to other methods
7) Less hazardous

Further on this point, what are you going to do about all the organs people and rodents have, but fish do not, and the converse? I will concede that the Stannius bodies and interrenal bodies are homologs of the adrenal cortex, the ultimobranchial body is homologous to C cells in the thyroid, and chromaffin bodies in fish are homologous to adrenal medulla, and so on, but what are you going to do about mammary glands (of which Minnie Mouse has not just 1 pair, but 5), lungs, trachea and larynx, uterus and placenta, and prostate? Cancers in these organs comprise over 50% of all cancers in man, but fish do not have these organs. Can the swim bladder, from which the lung is believed to have evolved, be a truly reliable indicator for lung carcinogens? Of course, somebody is going to throw the old quip at you that fish do not smoke. I once read a comment, in a discussion on the relationship between smoking and cancer, that it is remarkable that no lung cancer has ever been found in any invertebrate animal. Neither, of course does any invertebrate have a true lung. As with branchial and pharyngeal pouch derivatives, it would be interesting to know whether swim bladder carcinogens have a selectivity for mammalian lung or vice versa. Again, no one has induced cancers in swim bladder epithelium.

On point 2 (c) I have to admit that my client and all his relatives have failed miserably when it comes to developing skin cancer or lung cancer in response to arsenicals. Arsenical skin and lung cancers do occur in man. Can fish do something about this?

Point 3 overlaps slightly with 2 (c). It is an extremely tricky area. Many compounds are carcinogenic in rodents (e.g., 3-aminotriazole, griseofulvin, urethane, 2-fluorenamine, and the polychlorinated biphenyls), but we still do not know for certain that they are carcinogenic for man. Therefore, it is difficult to know what is a false-positive result. On the other hand, there seem to be few false negatives, arsenicals being the outstanding example. However, for your day in court with the decisionmakers, it would be well to have a tabulation ready, listing positive and negative results for fish versus rodents versus man, for every compound on which experimental and epidemiological data are available.

Point 4 deals with cost, a most important factor today, though it was less important when my client was rising to power. In 1976, Dr. Robert Squire (*3*) estimated that bioassay of a single compound required more than 1,000 rats and mice combined at a total cost of $200,000 to $250,000. Let us estimate that inflation has raised this figure to about $400,000. Can 2 species of fish in comparable numbers be tested at lower cost, leaving aside for the moment the questions of sensitivity, reliability, organ specificity, etc.? I have yet to see any hard information on this point, but you can rest assured you will be cross-examined on it during your day in court.

Now let us consider this: As far as I can see, the 1 organ on which all this enthusiasm for using Minnie Fish rests is the liver. Possibly kidney and gastrointestinal tract responses have shown some promise but nothing spectacular. Also, Dr. Kimura's report of chromatoblastoma induction in nibe offers possibilities not fully explored. How much is it worth to get information relevant to only 1 organ, or at best 3 or 4? If evidence is available on the pancreas, urogenital tract, hematopoietic tissues, soft tissues, bone, and all the rest, now is the time to bring it out.

If this information is not available now, the administrators are going to ask: "How much is it going to cost and how long will it take to get this background information on Minnie Fish? We have already done this for mice and rats, and it cost us a bundle. Suppose we go through this all over again with fish and we find out that fish are not that good or that cheap after all?" These are rough questions, but they will be asked.

In regard to point 5, speed has always been popular: in the air, on the ground, in the water, and especially in the laboratory. At the New York Academy of Sciences meeting in 1976, Dr. Stanton said: "The time factor is something I think can never be completely circumvented by any shortcuts, such as mutagenicity tests" (*4*).

Up to now, Dr. Mearl Stanton's opinion has proved pretty sound. In the ranking system proposed by Squire (*3*), this is where mutagenesis testing stands (table 2). At best, in Dr. Squire's estimation, it deserves a weight of 25 points, whereas mouse and rat tests rate 75 points out of 100. What weight should be given to positive results with fish? Remember that in establishing criteria for "sufficient" versus "limited" evidence, the IARC placed liver tumors in mice in the "limited" category, i.e., having lesser significance than neoplasms occurring at other sites. What does this say about the weight that would be given liver neoplasms in fish? My guess is that it would fall somewhere between the weights given to rats and mice, on the one hand, and genotoxicity tests on the other.

Suppose that a bioassay in fish could be accomplished in 6–12 months, or approximately one-half or even one-quarter the time required with rats and mice? All other factors being equal, this would represent a considerable advantage, but there lies the rub. All other factors are not equal, and if a positive result in 6–12 months only means that the same compound has to be further tested in mice and rats, one has only *added* 6–12 months to the time required if mice and rats had been used in the first place.

Being a generous Devil's Advocate, I would like to toss you a bone here. Because of the time and expense involved in the mouse/rat bioassay system, only a small number of candidate compounds can be tested, and these must be selected with extreme deliberation. It seems that if a fish system could be demonstrated to be "quick enough," "cheap enough," and "sensitive enough," it might merit

TABLE 2.—*Proposed system for ranking animal carcinogens*[a]

Factor	Score
No. of different species affected	
2 or more	15
1	5
No. of histogenetically different types of neoplasms in 1 or more species	
3 or more	15
2	10
1	5
Spontaneous incidence in appropriate control groups of neoplasms induced in treated groups	
<1%	15
1–10%	10
10–20%	5
>20%	1
Dose-response relationships (cumulative oral dose equivalents per kilogram body weight/day for 2 yr)	
<1 μg	15
1 μg–1 mg	10
1 mg–1 g	5
>1 g	1
Malignancy of induced neoplasms	
>50%	15
25–50%	10
<25%	5
None	1
Genotoxicity, measure in an appropriate battery of tests	
Positive	25
Incompletely positive	10
Negative	0

[a] Table is reproduced with the permission of the publisher (*3*).

deployment as a preliminary screening method. I think few would deny that if a compound is carcinogenically active in fish, it deserves testing in rodents.

This brings us to the question of how fish might be deployed to best advantage in the "decision point" approach to carcinogen testing as proposed by Weisburger and Williams (*5*). Because the fish system is a bioassay in vivo, it would not enter the picture until stage C (table 3). This is too late to save the costs of stages A and B. However, if quick, cheap, and sensitive enough, it is conceivable that a fish liver system could supplant mice or rats, or both, in stage C, under the heading of "altered foci induction in rodent liver." Inasmuch as mice and rats would have to be used anyway in a test for breast cancer and pulmonary tumor induction (stage C, items 2 and 3), these same rodent subjects might just as well be used as test subjects for altered foci induction in liver, *unless* the fish system is quicker or more sensitive, or both. This is a rather crucial consideration.

On point 6 in table 1, I have just given an example of when the time factor could favor supplementary uses of fish. However, I want to re-emphasize that any supplementary role means additional time and money. Therefore, the value received must be worth that additional time and money. I have said this might be true if a much larger *number of compounds* could be tested for a relatively small increment in time and money. This could also be true if a much larger *number of animals* could be tested per compound for a relatively small increment in time and money. For example, my client has been criticized because the numbers of mice and rats used per compound is nowhere near the number of human beings required in epidemiological studies to detect a population at increased risk to cancer. This is why maximal tolerated doses are used, on the assumption that large doses will compensate for low numbers of animals. Even so, weak carcinogens could be missed. The question boils down to this: Can 400 mice and rats (the number used in the final definitive test) beat 1,000, or 10,000, or 100,000 fish with respect to all of the points on this table? At what number level is the fish superior to rodents in relation to each point: sensitivity, tissue relevance, reliability, expense, speed, and hazard?

For the 400 mice and rats in the definitive test run, it costs $1,000 per animal because we have to include the costs of the preliminary toxicity tolerance tests. Can a fish beat this by 1 log or 2 logs, which might bring us into the range of human tumor incidences? This would mean that 4,000 or 40,000 fish could be used at the same price as 200 mice plus 200 rats. That is, the cost would have to be $100 or $10 per fish, respectively. I know it would cost more than $10 per fish, as this amount would not even cover the cost of the histologic sections at the end of the run. So let us say it costs no more than $100 per fish, a figure that I doubt, considering the time it takes a highly qualified though poorly paid pathologist to interpret the sections and write a report. Is a 4,000 fish bioassay as good as or better than a 400 mouse/rat bioassay? If not, you cannot hope to replace my client, and you are back to supplemental uses. Would a tenfold difference in cost per animal warrant supplemental uses? We all know the answer depends on how dire the need is, how much funding is available, and how good you are at convincing infidels.

TABLE 3.—*Decision point approach to carcinogen testing*

Stage A: Structure of chemical
Stage B: Short-term tests in vitro
  Bacterial mutagenesis
  Mammalian mutagenesis
  DNA repair
  Chromosome tests
  Cell transformation
Decision point 1: Evaluation of all tests conducted in stages A and B
Stage C: Limited bioassays in vivo
  Skin tumor induction in mice
  Pulmonary tumor induction in mice
  Breast cancer induction in female Sprague-Dawley rats
  Altered foci induction in rodent liver
  Assays for promoters
Decision point 2: Evaluation of results from stage A through all the appropriate tests in stage C
Stage D: Long-term bioassay
Decision point 3: Final evaluation of all the results. This evaluation must include data from stages A and B to provide basis for mechanistic considerations.

On the matter of hazards, let me only remind you of another thing you already know. Water is the great solvent and, with air, the great distributor of things on earth. Soluble and even suspendible compounds, whether carcinogenic or not, are eventually disseminated worldwide by water. We have good examples in dichlorodiphenyltrichloroethane and the polychlorinated biphenyls. In fact, this is one reason for my advocacy of studying aquatic animals as possible indicators of environmental carcinogens. Witness the problems encountered by nuclear medicine, nuclear industry, and nuclear warfare in disposing of radioactive wastes in the sea. Volatiles are delivered to the air, which completes the job of ultimate delivery to the biomass. The problem has been large even for my client, who has the tidy habit of depositing his excreta in an absorbent substratum of wood chips and not in a tankful of water which, up to the present, has gone down the drain into the handiest river or into some holding pond where the solutes find their way to the seas only slightly more slowly. Until now, it may be true that the amount of water pollution from laboratories in which fish carcinogenesis work is done is small, but, if work volume is increased, it will become necessary for us to follow the rule of handling every suspected carcinogen in a way that it could not be incorporated into the earth's aqueous or atmospheric pools. In mouse/rat assays, the carcinogens are inactivated by high temperature incineration, and the same would have to be done with fish bioassays except that there is the extra step of first removing the carcinogens from the water or chemically inactivating them. This step adds expense.

I find it a bit alarming to see investigators doing carcinogenesis experiments with fish and discharging the water into standing earth ponds. A compound is never fully retrievable from that kind of situation. Worse yet, it is next

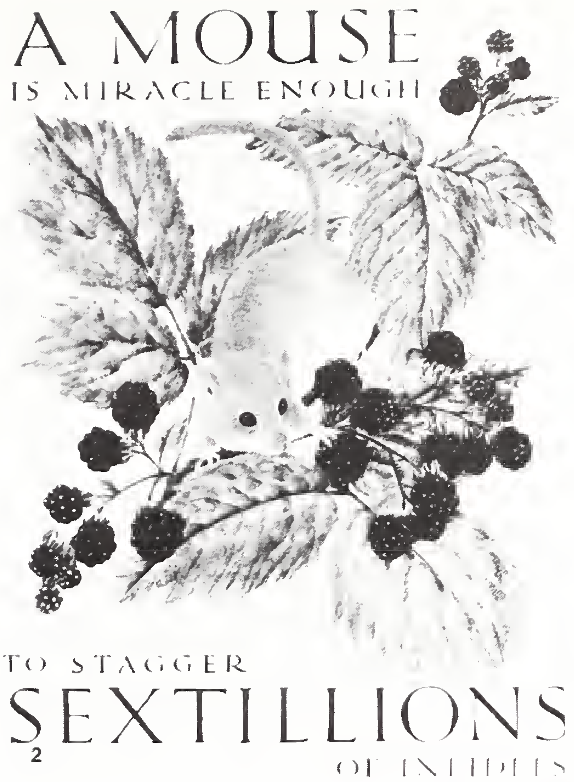

FIGURE 2.—Title is that of a poem by Walt Whitman. Figure is reproduced with the permission of the Green Tiger Press, La Jolla, California.

to impossible to trace, unlike radioactive materials. I think a real chance exists that those who work carelessly with carcinogens in aquatic systems may find themselves hoisted on their own petards. Take a hint from the experience of my terrestrial client.

Have patience, I am about to conclude. My message is simply this: It pays to know the enemy, even if, in the famous words of Pogo, "We has met the enemy and he is us." What we are trying to achieve is an understanding of the weaknesses and strengths of two systems, so that the best aspects of each can be used to greatest advantage. I think that on Judgment Day neither Mickey Mouse nor Minnie Fish will be identified as Angel or Devil. They have a little bit of both in them. Even carcinogens can work for good or evil: Estrogens can turn innocent little cells into devilish cancer cells, but they can also turn devilish little girls into angelic women. Because I have had to keep a thread of sex in this sermon to hold your flagging postprandial attention, I will conclude with this quotation from Walt Whitman, a wise poet who, with Robert Burns, was a great admirer of mice: "A mouse is miracle enough to stagger sextillions of infidels" (fig. 2).

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II. THE FISHY SIDE: *JOHN A. COUCH*

Clyde Dawe pointed out that he is playing the Devil's Advocate here and addressing a congregation of believers. I would like to carry his analogy one step further. In any congregation, you not only have the devout, perhaps to the point of fanaticism, but you also have your share of skeptics and doubters, some to the point of agnosticism. Actually no one pays much attention to the few village atheists that are around.

However, each of you here have your views and convictions concerning the relevance of fish and aquatic animals in general as test organisms and as models. I hope that the papers presented to date in the Symposium, and those to be presented, will permit all of us a more enlightened examination of our views and beliefs in regard to fish versus mammals as experimental animals, as well as a further appreciation of the unique value of fish.

I must yield, at the onset, one important point in my attempt to defend the use of fish as carcinogen test organisms: We are obviously not dealing with an established, standardized technology. We are in the process of developing this technology, and I think the rodent oncologists can admit a great headstart on us in that regard. Despite their headstart, as Dr. Dawe has pointed out, the rodent oncologists, after 50 years or more, frequently find themselves in the same boat that we are in with fish, i.e., facing certain longstanding enigmas relating to the peculiarities of their subject. Perhaps that is a warning to us about insoluble problems. We can benefit, of course, from their experience with what problems should be attacked more readily and where to spend our zeal and our funds.

Much of how we value fish as test animals depends upon our disciplinary perspectives, which, in turn, are moderated by our experience with limited numbers of species and agents.

Thus I hope that you will bear with me as I present my personal view of the advantage and value of using fish as carcinogen test animals. I also want to include the statement, perhaps apology, that I will not attempt to set the fish as a replacement, in my scheme of reasoning, for the mouse or the rat. This should become apparent.

In figure 1 (*1*), we attempt to depict the fundamental concepts of biological overlap (relatedness) and uniqueness among living species. Scientifically this is an expression of faith in theory. We biologists or life scientists generally tend to accept with evidence, and intuitively perhaps, the idea that there are common threads among all living forms. I call this "biologic commonage." Perhaps it is more evident in the characteristics that are listed in the right hand column at the cellular level and below. As an example, if we look at the mitochondria of protozoa and of man, we may find a few qualitative but mainly quantitative differences in their structure and function. Essentially, the form and function are similar, that of cellular respiration. If you apply a poison such as cyanide to a protozoan mitochondrion, cytochrome oxidase *a* probably will be inhibited essentially the same way it will be in the mitochondrion of man. So at the molecular level at least, I believe that we have a conservative basis for extrapolation from fish to mammal based upon what Dr. Dawe has referred to as the publicly unpopular evolutionary theory.

I think evolutionary theory has never been too popular with the general public; that is not a new problem that we deal with as scientists. To have the foundation for the understanding of life forms and to realize that this chain or this relationship (biologic commonage) exists among forms at certain levels requires a great deal of experience, thought, and a broad view in dealing with life forms. In that regard, it is a view that life scientists use as a working point of departure but one which may not be widely appreciated by the public.

We could call forward a great deal of factual evidence to defend this chain of life concept and present it as the basis for extrapolation of the significance of response at one phyletic level to that at another phyletic level. It is partly on these theoretical considerations that I will attempt to build a case for fish as good experimental, predictive models.

I would like to go on to a couple of objectives that support my personal viewpoints as to why I work with fish as carcinogen test organisms. They will differ from many of yours, perhaps, and I may omit some points; again, they represent my personal view. As much as anything, this is a statement of what we hope to do and perhaps of what some of the results will be.

The first objective is that we want to develop fish test systems that characterize sensitivity of different species in regard to detecting and determining potency and activity of carcinogens. This will result in practical bioassays with fish as well as expansion of comparative studies in cancer research. In doing this work, novel carcinogen screening methods should be found serendipitously. I think that we can depend on certain methods coming to light that we have not even anticipated. This is one characteristic of science; as Louis Pasteur said, or I will paraphrase: Chance favors the prepared mind.

Fish tests are complementary and supplemental extensions of existing vertebrate test systems. This goes along with what I said earlier; they are not a replacement for rodent or mammalian systems.

The second objective, in my view, follows from the first. From knowledge gained in laboratory and field studies, we should be able to predict and verify responses of representative fish species to carcinogen exposure in the environment; this increased capability would stem from our greater understanding of comparative carcinogenic mechanisms in these animals. The effort should permit linkage of cause and effect in the larger environment and underlies one of the key problems with which I am concerned. If fishes or aquatic organisms are of any value as indicators or sentinels, I think that they may eventually enable us to link

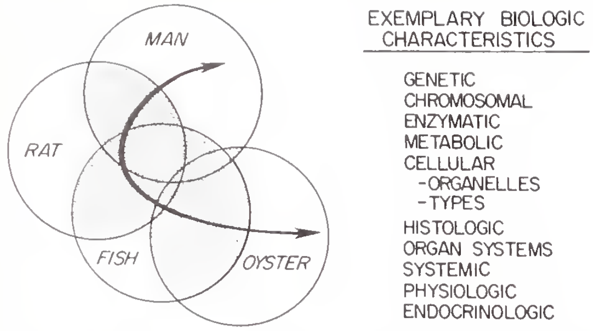

FIGURE 1.—Biologic commonage suggested in an analogy of circles through which runs a double-headed arrow of continuity. At the levels of form and function suggested in the right-hand column, biological similarities may be most real in animals from different phyletic levels. *Shaded areas* indicate overlap or shared characteristics; *open areas* indicate biologic uniqueness for each species. Adapted from (*1*).

cause and effect in the larger, natural environment, warning us of unacceptable risks to humans from certain agents in the environment.

I am going to do a dangerous thing: I have put down a number of ways in which the usefulness of fishes may be compared with that of rodents, particularly mice. This comparison presumes to show where fishes exceed, or are equal in comparison with rodents as carcinogen test animals (table 1). I want to go through these obvious comparisons of fish and rodent systems with you, and perhaps at the end, we can have some open discussion from the floor, if you wish, and you can point out some of these things that I have omitted.

Point one (table 1) states that fish are readily available, relatively easy to maintain, house, and manipulate experimentally. (Everything here is set in comparison with, and in contrast to, rodent or mammalian test animal populations.) They are adaptable in maintenance and in the conducting of tests.

TABLE 1.—*Presumed obvious advantages of fishes as carcinogen assay organisms*

Indicative symbol[a]	No.	Advantage
>	1)	Fish are available and relatively easy to maintain, house, and manipulate experimentally.
>	2)	Fish are inexpensive (relatively).
=	3)	Some species show as good response, or are even more sensitive in their response, as mammals (agent-specific).
>	4)	Fish live in water, a relatively efficient solvent and carrier.
>	5)	They are intimately dependent upon and in constant contact with ambient medium; at minimum, superficial exposure by contact is assured.
>	6)	Due to general and specific distributions of fish populations in relation to real world pollution or contamination, many a priori in situ experiments await the astute investigator in the natural (?) environment.

[a] > indicates advantage is superior to rodents; = indicates advantage is equal to rodents in usefulness.

In reference to housing your experimental animals, Koenig and Chasar (*2*) have shown us a fish condominium–high-density housing, for their experimental species. In their paper, you can see *Rivulus* stacked vertically in dishes. *Rivulus* is essentially a desktop assay setup or a potential one. *Rivulus* is a fish that is easy to maintain with minimum requirements in support systems.

From desktop or bench, we go up the scale a little in facilities, and arrive at a fish assay laboratory that consists of a small, permanent building (~160 ft^2) as a useful, isolated test facility (fig. 2). One may add flow-through water for better maintenance of certain fish in this situation.

I suppose you could work with rodents in small laboratories, but I suspect that you would need a good deal more space than this (fig. 2) to be practical. We have held and conducted tests with several hundred specimens of a given species of small fish to be used with specific chemicals in this type of facility.

If mobility in combined laboratory and field efforts is required, one can have a custom-built trailer which can be moved from site to site in the environment. Another advantage of working with fish is that, if you are concerned with specific pollution sites or those where agents might be present in water, you can move the trailer to a site for any period and do the short- or long-term exposure tests with mixtures from the environment with pumped-in water.

Ultimately you can go up to the Xanadu or the Superdome of fish experimental wet laboratories, which we consider to have been recently constructed at the Environmental Protection Agency Laboratory at Gulf Breeze, Florida (fig. 3). Thus one may envision a facility where almost anything can be attempted with fish, depending on the availability of funds and personnel. Many existing aquatic laboratories can presently provide basic facilities.

Let us talk a bit about the second category, *expense*. Expense, of course, is largely dictated by the size of the experiment, i.e., the number of animals used and end points measured.

Dr. Dawe has suggested that one would need to assay at least 4,000 fish/test to give a statistical edge to fish as carcinogen test animals. From a pragmatic point, that would probably be impossible due to such problems as personnel safety, disposal of contaminated food or water, etc. I would guess that no more than twice his estimate of mouse or rat numbers (i.e., 2×400=800) would be practical for the largest of present fish assays for a given compound or agent. This would assume the use of a small fish species. Furthermore, carcinogen tests utilizing embryos or fry of fishes in short pulse exposures, followed by grow-out without further exposure, could increase the numbers of fish used (*3, 4*) and reduce the amount of waste water generated. Static pulse exposure of small fish in finite amounts of water would prevent release of any waste to the environment.

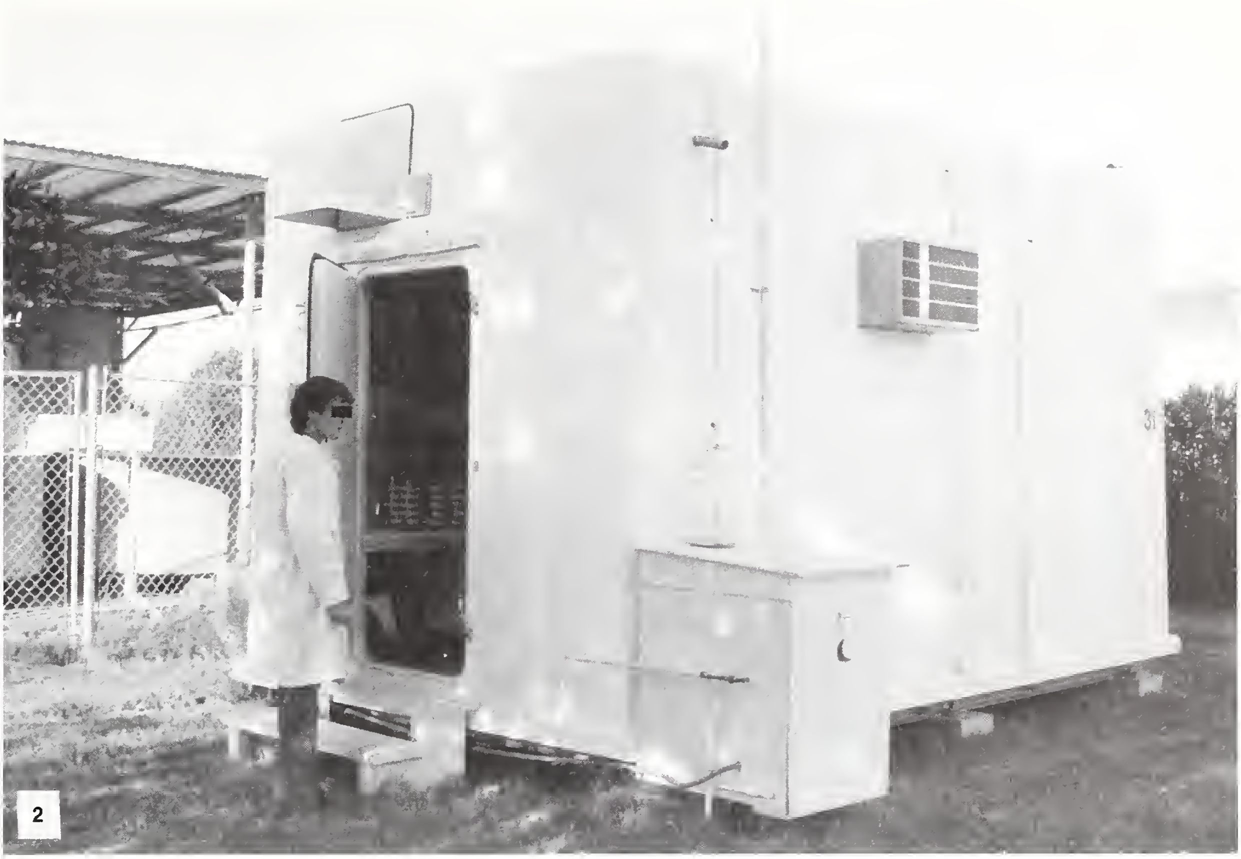

FIGURE 2.—Small aquatic laboratory for conducting fish carcinogen assays in isolation with minimum of space. Static or flowing water tests can be conducted in such a facility.

Our exemplary test at Gulf Breeze was concerned with the herbicide trifluralin, which was once identified as a suspect carcinogen. We actually exposed fish for 20 months and made observations up to 36 months with several hundred experimental and control specimens in the test (with about 400 fish; larger numbers could have been used). We estimated the cost to be $80,000 to $100,000 (table 2). Incidentally, we found trifluralin noncarcinogenic to fish in our system, but the compound did induce significant non-neoplastic lesions (*4*).

The costs for rodent tests for a single compound, with multiple observational end points, including many of the end points that Dr. Dawe showed on the table from Weisburger's article (*5*), is between $300,000 and $500,000. We took 36 months with fish but the test period might be only 24 months to obtain similar data. The 64 months was Weisburger's figure for rodents (*5*).

Let us discuss the third point (table 1), *sensitivity*. In high performance liquid chromatograms supplied by Schoor (*6*), the biochemical or metabolic response of metabolites of BP produced in the liver of mullet (a marine fish that was injected with the parent compound following induction) reveal that the mullet metabolic profile is similar to that of some rodents. Most of the major metabolites that have been identified for BP in rodents are produced by the fish qualitatively.

In certain cases, fish quantitatively respond in the production of certain metabolites in ways comparable to certain of the rodents. Some fish, on the other hand, do not respond quantitatively as the rodents do. As an example, Dr. Melius (*7*), using S-9 preparations, presented us with a comparison of BP metabolite formation by the mullet with that by the rat. He demonstrated that the major metabolites of BP are similar in fish but are only produced at about one-fourth the metabolic rate of rodents. Therefore, a great deal of similarity is observed in the response of the animals in the qualitative metabolic response to certain carcinogens that have been tested.

With tumorigenesis as the end-point response in rats, Wogan (*8*) studied the potency of aflatoxin and reported that the total numbers of hepatic carcinomas in rats are related to the aflatoxin concentrations administered. Thus in rats the carcinogenic response to aflatoxin is dose dependent.

Rainbow trout also respond to aflatoxin in a dose-dependent manner. Hendricks et al. (*3*) found trout to be considerably more sensitive than rats, both in numbers of tumors produced and latency for tumor production. Mice, on the other hand, show no tumorigenic response to aflatoxin at all.

In regard to this specific compound, trout demonstrate more sensitivity than do the standard rodents used in

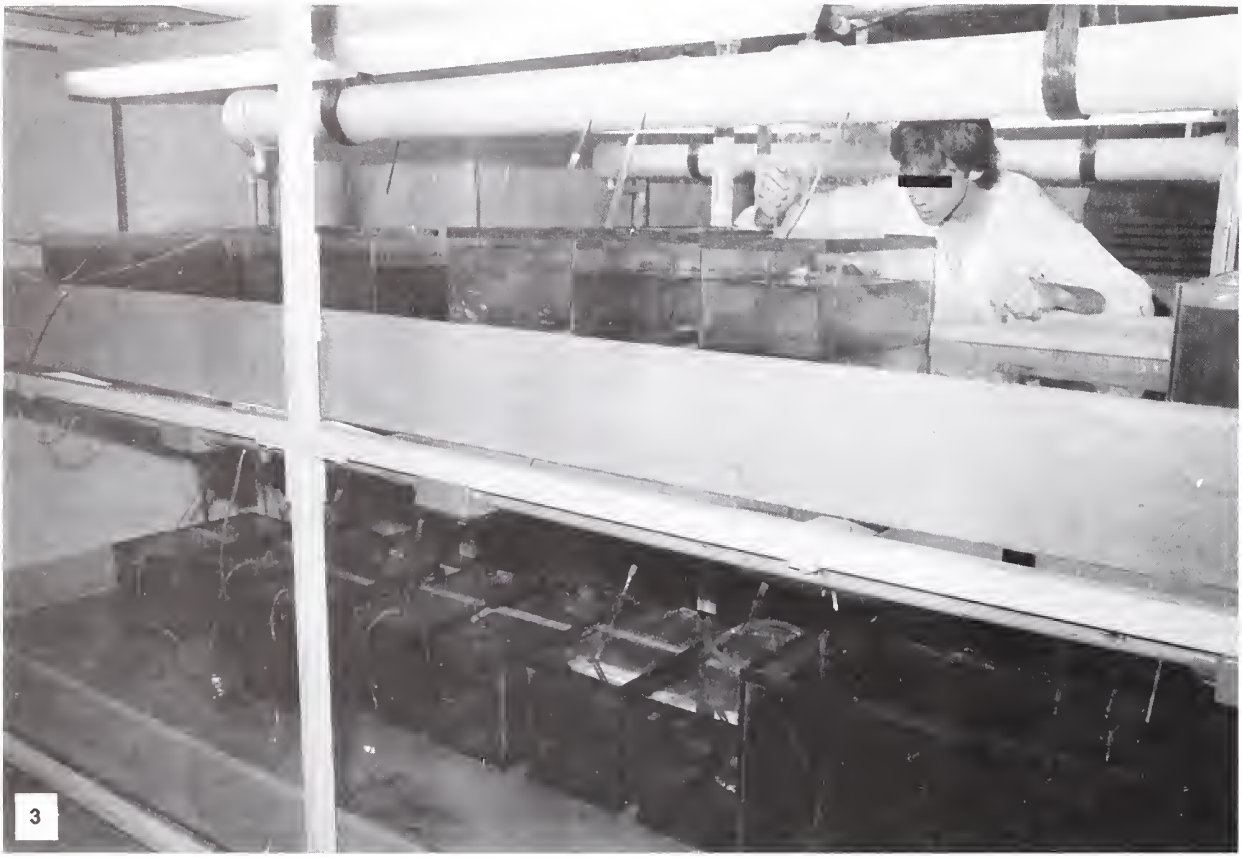

FIGURE 3.—Larger wet laboratory facility, designed for the safe use of fishes in carcinogen-mutagen testing (Gulf Breeze Environmental Research Laboratory, Gulf Breeze, Florida).

bioassays. We also must remember that with aflatoxin it was largely the research with trout that led to the identification of the mycotoxins as carcinogens, although a few turkeys participated in that, too, in Europe. For most practical purposes, many people here believe that it was mainly work with trout that led to the identification of this group of compounds as carcinogens.

It is interesting, also, as Dr. Dawe pointed out, that Dr. Kimura (*9*) has come up with the second compound (nifurpirinol) to be implicated as a new carcinogen, solely from fish work.

Let us proceed to points 4 and 5 in table 1. I think we deal with truisms and cliches in everything we do, and, sometimes because they are truisms or cliches, we tend to ignore their significance. Fish live in water, an efficient solvent and carrier, which Dr. Dawe graciously pointed out in his presentation. Fish are in constant contact with their ambient medium and, unlike terrestrial animals, possess physiological mechanisms intimately dependent on the physical and chemical conditions of their surroundings. They have little choice, as many terrestrial organisms do, to move about and escape an air mass or irritant quickly, for example, the way birds and even terrestrial animals can.

TABLE 2.—*Single chemical test cost estimates for carcinogenicity testing in rodents and fishes*

Species	Multiple observational end points	Cost in dollars (× 1,000)
Rodents	Up to 64 mo, several species	300–500[a]
Fishes	Up to 36 mo, 1–3 species	80–100

[a] Data are from (*5*).

Fish may provide a more efficient field and laboratory model for exposure to carcinogens. We can depend on water, if not for being the perfect solvent for any compound to which we want to expose our animals, at least for being a carrier. In the laboratory, one may use any number of solvents as vehicles and carriers to get a suspect carcinogen into water and thus into contact with the fish. Marine fishes are interesting because they continually drink sea water, thus insuring some internal exposure to test chemicals dissolved in or carried by their ambient medium.

Another point concerning item 5 (table 1) is exemplified by problems with potential assays with which one determines the effects of low concentrations of suspect carcinogens in drinking water around the country. The relevant question may be asked: Could you pump enough water with low carcinogen concentrations through the gut of a mouse in its lifetime to assure an exposure that would result in an indicative tumorigenic response? You know that you can put the fish in water; there is no escape from exposure

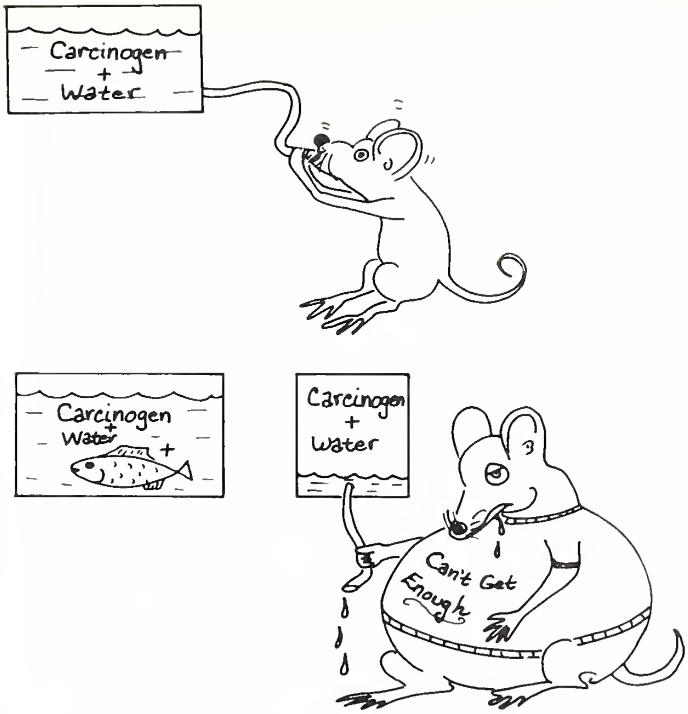

FIGURE 4.—Long-term test exposure of subject to low carcinogen concentrations may be more effective with fish in water-carcinogen mixture than with mouse attempting to drink enough carcinogen over its lifetime to accrue an effective dose.

for them; because we can hold large numbers of fish up to 36 months or longer, we could use environmentally realistic, sublethal concentrations of suspect chemicals in simulated drinking water mixtures for long-term experimental exposures, assuring simple, direct exposure and continual contact (fig. 4).

Now let us look at item 6 in table 1. Due to general and specific distributions of fish populations in relation to real world pollution or contamination, many in situ experiments await the astute investigator in the natural environment.

I think the work of Dr. Kimura and his associates (*9*) is a good example of a phenomenon in nature in which a high prevalence of a certain tumor type was detected in a feral population of fish, along a coastal area, the cause of which was then investigated with detailed laboratory studies. Kimura et al. showed that the chemical nifurpirinol could induce this same tumor type in the same species of fish brought in from nature, which provided evidence of a possible chemical etiology.

Many of you who work only in the laboratory may not realize the considerable potential that observations from field studies can bring, but because of the increasing numbers of polluted harbors and estuaries, others have experiments waiting to be done in nature. The critical results from these studies will probably depend upon the ingenuity of the investigators and their ability to design an adequate and valid experiment. Some of the less obvious advantages of using fish as carcinogen test animals are given in table 3.

Point 1 has an equal sign because I believe this could apply to any rodent or wildlife population, including fish, but not necessarily to an inbred, highly selected group of laboratory animals. This point is debatable and controversial. However, results of a test can be highly significant as warning of risks, if you are dealing with an effect relevant to the real world because it occurs in individuals from natural populations of organisms. In this case, we are dealing with wild fish. We have found that many times, if we expose these genetically variable fish to an agent in the laboratory, we get an either/or pathological response, making close statistical decisions unnecessary and the response to some degree independent of genetic control as a deciding factor.

Tumor induction work done with even more domesticated fishes, such as the rainbow trout, often demonstrates this either/or response in which background or control incidence of tumors is usually zero, and tumors that occur in the exposed animal are related to an experimental variable, usually the agent that was treated. On the other hand, it may be desirable, and possible sometimes, if one selected fish with enhanced genetic proclivity for neoplasia (table 3). The work of Dr. Anders (*10*) has helped identify such fish strains, particularly the *Xiphophorus* system. Therefore fish, as good vertebrates, are not at a disadvantage in comparison to rodents as variable or restricted genetic test substrates.

Point 3 (table 3) is one that I am convinced about and think is one of the major advantages in using fish as model systems in carcinogen assay studies: Critical life stages, e.g., embryos, are easily handled, manipulated, observed, and externalized, usually without compromising natural de-

TABLE 3.—*Less obvious advantages of fishes as test animals*

Indicative symbol[a]	No.	Advantage
=	1)	Use of heterozygous fish of wild ancestry may reduce need for close statistical decision because tumorigenesis reflecting a real world risk probably is an either/or response and not dependent on a genetically selected test animal that may possess an enhanced susceptibility to neoplasia.
=	2)	In specific, desired instances, fishes with some enhanced susceptibility to neoplasia may be selected for experimental purposes.
>	3)	Critical life stages (e.g., embryos) are easily handled, manipulated, observed, and externalized without compromising natural development. Fish can be directly exposed from zygote to adult.
>	4)	Fishes can be cloned.
>	5)	Whole-body, serial sections can be mounted on slides for complete study (slide) of small fish and embryos.

[a] *See* explanation, table 1 footnote.

velopment. Fish can be directly exposed to carcinogens from zygote to adult. I think there are few mammalian systems that can approach that utility.

We learned at this meeting from Dr. Streisinger's work (*11*) that individual fish now can be cloned (point 4); this is a starting point for doing research with genetically identical fish, i.e., homozygous, diploid individuals, and supports points 1, 2, and 3 in table 3.

Examine point 5. Whole-body sections can be mounted on slides for complete serial sections of the whole animal, if desirable, of fry and young fish, small adult aquarium fish, embryos, and other developmental stages. I think this whole-body approach with small fish also is true for radiographic studies, in which one may radiograph a large number of small specimens on a single film plate. Perhaps one could do this with mice too, but overall, I think the manipulative utility is superior in fish.

Courtney and Couch presented a photograph [fig. 7 in (*4*)] which shows a *Cyprinodon* fry, a young fish, in a longitudinal, horizontal section; one may examine, in serial sections, all the organ systems, from dorsal to ventral planes, with ease. These sections can be examined at relatively low power for conspicuous lesions, particularly tumors. One can magnify relevant tissues to fifty times by using microviewer screeners (or microfiche readers) to examine slides; therefore, large numbers of fish could be screened histologically (fig. 5).

Figures 5 and 6 in the paper by Courtney and Couch (*4*) illustrate sections of a fish embryo with major tissues and organs visible: The yolk sac, the oral cavity, and the branchial chamber, the gill clefts, the well-developed massive brain tissue, and the proportionately large liver are all visible. These sections permit one to examine critical tissues for lesions at an early developmental period following exposure that might lead to full neoplastic development later in life or those that are already at that point. Their potential for manipulation and examination make fish superior experimental animals in comparison to most mammals.

Let us examine the potential use of fishes within the "battery of tests" recommended by Weisburger (*5*) in his "decision point" approach to carcinogenesis testing in general. In regard to short-term tests, we already have the use of bacterial mutagenesis (screening test) with fish preparations, particularly in conjunction with the use of fish S-9 preparations for activation of mutagens. As Ishikawa and associates (*12*) indicated with the medaka, DNA repair studies are possible with fish as a way to screen the possible effects of chemicals. Also, development of a sister chromatid exchange assay with the use of 22 large, clearly observable chromosomes of the mudminnow (*Umbra* sp.) promises to be a useful screening technique in both field studies and short-term bioassays of suspect carcinogens within the laboratory (*13*).

Skin tumor induction in experimental fish has been reported, but, as Dr. Dawe mentioned, at this point the

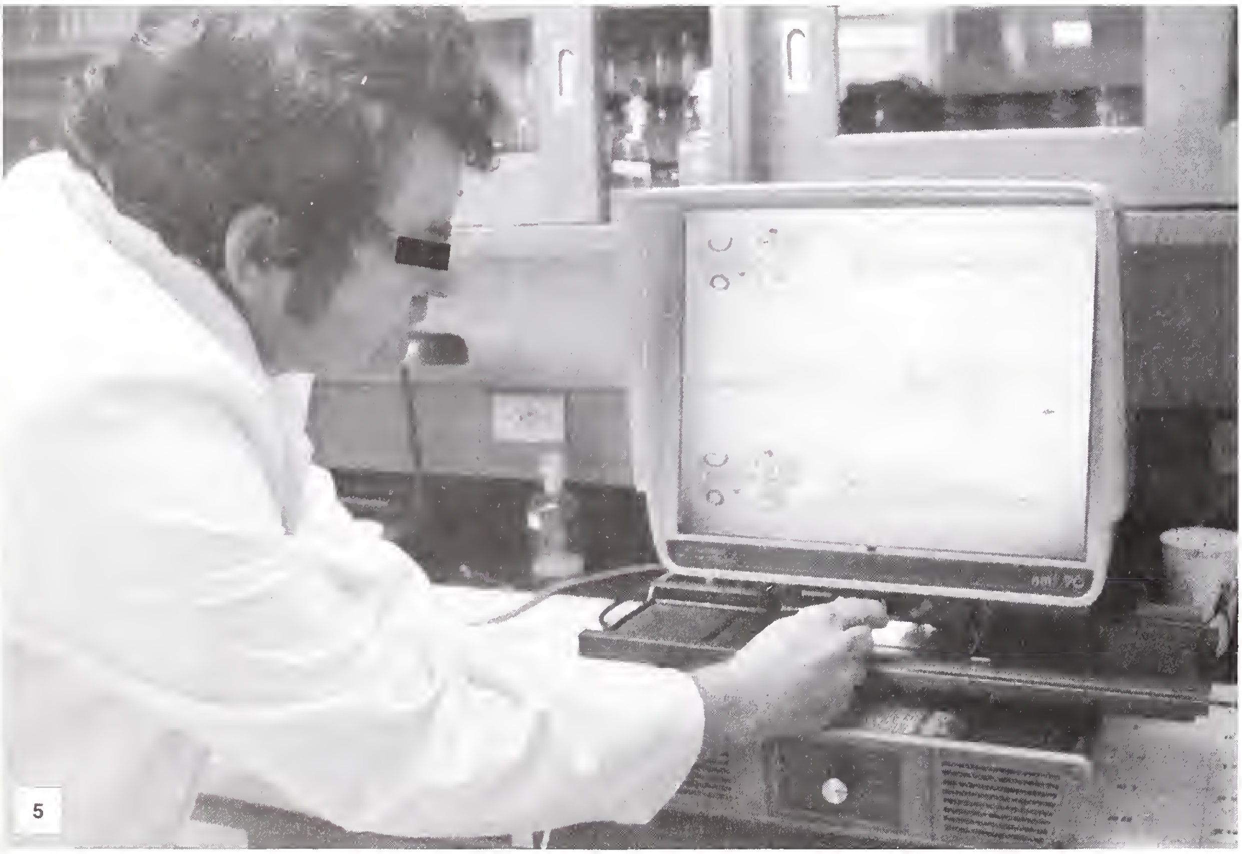
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FIGURE 5.—Use of microviewer (×50) expedites screening of whole-body serial sections of small fish for lesion detection.

liver seems to be the most useful assay tissue for fish experimentally exposed to a suspect carcinogen in the laboratory. Tumor induction in many other fish tissues may be observed as more studies with other agents are completed. Already, tumors have been experimentally induced in the kidney, alimentary tract, and tegument of some fishes.

If one examines the accession records at the Registry of Tumors in Lower Animals, one sees that fish spontaneously develop nearly every kind of tumor that has occurred in comparable tissues within mammals, e.g., tumors ranging from those in teeth to the kidney to lymphomas. Fish are no more fortunate than mammals in being victims of varied neoplastic disorders.

As we have heard at this meeting, long-term bioassays with fish are already being done by several investigators. Perhaps we are going to have to retrace our steps and emphasize some of the earlier screening tests just mentioned in assessing the usefulness of such assays, before we initiate them. We should carefully consider the points made by Dr. Lech (*14*) concerning the presence or absence of target tissues, as well as inherent sensitivity, in our selected fishes.

Essentially, I have tried to present fishes not only as laboratory animals but also as indicator organisms in the environment. I have my doubts that either approach, field or laboratory, can exist independently of the other in the most efficient utilization of fish as carcinogen test animals.

Finally, I have attempted to put in perspective the need for fish carcinogen test systems. This is summed up in the deathless quote of one of our esteemed colleagues: "You're going to have a heck-of-a-time training a mouse to swim upstream in a polluted river."

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Session IV

Special Problems and Topics Related to the Use of Fish in Cancer Research

Chairman: **Jerry M. Rice, Ph.D.**

Effect of Age and Exposure to a Carcinogen on the Structure of the Medaka Liver: A Morphometric Study[1, 2]

David E. Hinton, R. Clark Lantz, *and* James A. Hampton[3, 4]

ABSTRACT—Morphometric evaluation of liver in the Japanese medaka (*Oryzias latipes*) was done to define components quantitatively. Analysis was performed in individual fish at: day of hatch (larval), 3–6, and 6–9 months, and 1 year. From analyses of hematoxylin and eosin-stained paraffin sections, the volume densities of hepatocytes, blood vessels (inclusive of sinusoids), intrahepatic bile ducts and ductules, and other components (connective tissue, melanomacrophage centers) were determined. No significant age-related changes were seen. To determine those structural parameters which undergo change 66 and 116 days following a 10-day exposure to methylazoxymethanol acetate (0.5 ppm in water), we performed analyses as above on livers of treated and paired control fish. The stained paraffin sections demonstrated a significant decrease in the volume densities of hepatocytes and blood space and an increase in volume density of bile ducts 66 days after exposure. Although these whole liver values returned toward normal 116 days postexposure, significant alterations in hepatocytes were still seen when toluidine blue-stained semithin sections of Epon-embedded liver were studied. The nuclear-to-cytoplasmic ratio was increased as was stainable material at the cell periphery. The most dramatic change was in the number of hepatocytes per cubic centimeter of liver which was increased by 300% over controls in animals 116 days post-exposure. Morphometric data were compared with subjective evaluation at the light and electron microscopic levels. — Natl Cancer Inst Monogr 65: 239–249, 1984.

Tumors of the liver appear in small aquarium fishes exposed to water-soluble carcinogens (*1–5*). The short period of induction, relative ease of handling, and reduced expense of exposure of large numbers of these animals coupled with the need for a suitable animal model for analysis of carcinogenic substances in water favor the development of small aquarium species as meaningful organisms in aquatic carcinogen research (*6, 7*). Although several compounds are used to produce tumors, tissue alterations appear to follow a pattern partially worked out in some species (*1–5*). Diagnosis of tumor is based on gross observations of liver, morphological properties of hepatocytes, patterns of arrangement between adjacent hepatocytes, and involvement of bile ducts and ductules (*1–5*). Because these results appear promising, more thorough knowledge is necessary, in quantitative terms, of both the normal structure of the liver in these fish and the alterations which the liver undergoes during the development of the neoplastic process. When larval stages of fish are exposed to concentrated carcinogen, tumors result within a few months (*8, 9*). This young age of tumor-laden fish requires that our knowledge of base-line liver structure in these species be extended to the understanding of changes that may occur in the normal liver with age. We present our findings on liver in control medaka from larva through adulthood and those in medaka exposed to the chemical carcinogen MAM acetate. Multistage morphometric analysis is useful in gaining quantitative structural information concerning organs, tissues, and cells (*10–12*). Such data can be correlated with chemical and physiological studies (*12*).

MATERIALS AND METHODS

Fish.—Adult male and female Japanese medaka (*Oryzias latipes*) were obtained from the Carolina Biological Supply Company (Burlington, N.C.) as breeding stock. Using the recommended procedures for care and maintenance of this species (*13*), we established a breeding colony from which randomly selected fish were taken. Fish were maintained at room temperature (22° C) in 10- and 40-liter aquaria in aged water (pH 6.8–7.0) that was dechlorinated and continuously aerated. Fish were maintained on a 12-hour light/dark cycle and fed Tetramin daily.

Compounds.—A water-soluble carcinogen, MAM acetate was obtained from the Aldrich Chemical Company (Milwaukee, Wisc.), dissolved in sterile water, and stored frozen until initiation of exposure.

Age studies.—We assessed the effect of age on liver morphology by taking fish immediately after hatching and at 3-month intervals up to a year of age.

Exposure to MAM.—Randomly selected fish, 3 to 6 months of age (1–2 cm in length), were placed in containers

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ABBREVIATIONS: MAM = methylazoxymethanol; LM = light microscopy; H & E = hematoxylin and eosin; HRLM = high-resolution light microscopy; TEM = transmission electron microscopy; V_v = volume density; N_v = numerical density.

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[2] Supported in part by the Water Research Institute, West Virginia University, with funds allocated under the Water Resources Act of 1964 (PL 88-379), administered by the Office of Water Research and Technology, U.S. Department of the Interior, as part of Project A-037-WVA; and by grant IN-127-A from the American Cancer Society.

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[4] We thank Dr. Roger Ulrich, Mrs. Elsie Syner, Mrs. Diane Rosnick, and Dr. Lyndell Millecchia for their technical assistance and Ms. Donna Borland for her help in preparation of the manuscript.

(750 ml) with either 0 (control, $n=8$) or 0.5 ppm (treated, $n=12$) MAM acetate in water. Following a 10-day exposure, fish were removed from the containers, placed in 10-liter aquaria containing fresh water, and fed daily. All procedures of exposure and handling of MAM acetate or MAM-treated fish were conducted under an appropriate hood. The 750-ml of contaminated water was placed in marked containers for subsequent disposal. Control and treated fish were killed at 66 (control, $n=4$; treated, $n=4$) and 116 (control, $n=4$; treated, $n=8$) days after exposure. Livers were dissected and processed for qualitative and quantitative evaluation at both light and electron microscopic levels. Remaining tissues were also processed for LM. Each fish was sexed by histological examination. No mortality occurred in either control or treated fish up to 116 days after exposure to the compound.

Necropsy procedures.—Live fish were removed from aquarium water and placed directly in fixative solution. Following cessation of movement, fish were transferred in petri dishes to a dissecting microscope for gross observation and tissue removal.

Tissue processing.—For LM, tissue from all groups was placed in Dietrich's fixative (30% ethanol, 10% formalin, 2% glacial acetic acid) for 48 hours, embedded in paraffin, sectioned at 6 μm and stained with H & E.

For HRLM and TEM, 116-day treated and control fish were placed in one-half strength Karnovsky's fixative in 0.1 *M* phosphate buffer (pH 7.4, 290 milliosmoles). Intact livers were dissected, placed in a drop of one-half strength Karnovsky's fixative, and sliced approximately in half. One piece was transferred to Dietrich's fixative and processed for LM (*see* above). The other piece was gently minced, rinsed in phosphate buffer, postfixed in osmium tetroxide, stained en bloc with uranyl acetate, dehydrated, passed through propylene oxide, and embedded in Epon-Araldite. Thick sections (1 μm) were cut and stained with toluidine blue (*14*) for HRLM. Thin sections (gold to silver interference color) were doubly stained with uranyl acetate (*15*) and lead citrate (*16*) for qualitative TEM with a 100 CX electron microscope from JEOL USA (Medford, Mass.).

Morphometry.—With standard multistage morphometric techniques, utilizing point counting as previously described for the rat liver (*17*), we evaluated the percentage of total volume occupied by various compartments (V_v), surface area/unit volume (surface density), and the number/unit volume (N_v) of tissue at the light microscopic level.

In the first stage, H & E-stained sections of paraffin-embedded material at a magnification of × 300 were analyzed for the determination of V_v of hepatocytes, bile ducts, blood space (including sinusoids), and all other components (connective tissue, melanomacrophage centers). For the second stage of the analysis, 5 of 10 Epon-Araldite blocks from each liver were selected at random, sectioned, and stained with toluidine blue. Our analysis (× 1,560) was performed to measure V_v of hepatocytes and cellular components of hepatocytes (nucleus, nucleolus, plasma membrane, lipid, clear areas, dark-staining regions of cytoplasm), surface density of hepatocytes and N_v of hepatocytes. The N_v was calculated from the equation:

$$N_v = (K \times N_a^{3/2} / V_v^{1/2} \times \beta),$$

by assuming that each hepatocyte contained a single spherical nucleus; N_a is the number of nuclei/unit area on the sections and V_v is the volume density of hepatocytes in the liver. The variable, K, depends on the distribution of sizes of structures being counted and for a single-sized structure $K=1$. When multiple-sized structures are counted, setting K equal to 1 will introduce at most a 10% error. Therefore, K was set equal to 1 in our calculations. The β depends on the shape of the structure being counted, and for spheres is equal to 1.38. At both levels, the test grid used for point counting was a 10 × 10 square lattice (total points/field = 100). The distance between points was 16 μm for × 300 and 3.2 μm for × 1,560, respectively. Two fields/sections, 5 sections/animal were evaluated. We collected the data using a semiautomatic computer-assisted device from Micrographics, Inc. (Cuyahoga Falls, Ohio) and stored the findings on disks for future analysis. Differences between groups were tested by analysis of variance with $\alpha=0.05$.

RESULTS

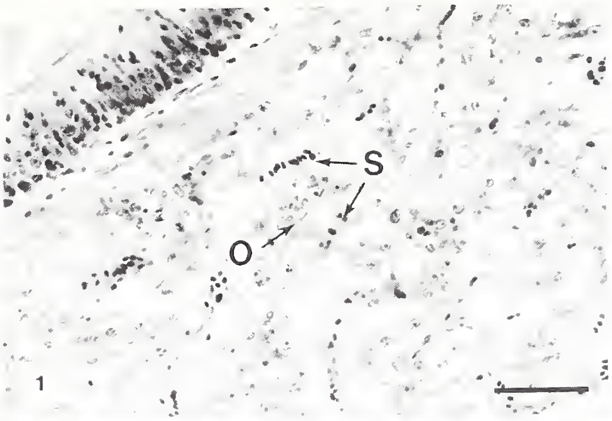
Gross Observations

No changes in growth, feeding, or behavior of the MAM-treated fish were observed. Before they were killed, treated fish commonly showed distended abdomens, their livers were pale, brownish-red, and under the dissecting microscope were seen to contain small pigmented foci. In some, vessels surrounded circular, less vascularized areas which suggested nodularity. Grossly observable liver nodules were seen in 1 of 5 fish at 66 days after exposure but not in the 116-day group. Control livers were smooth, pale brown in color, and usually showed a uniform vascular pattern.

Qualitative Observations

Control

Sections under LM (fig. 1) and HRLM (fig. 2) from control fish showed the typical dual-plated muralium described as a tubulosinusoidal liver (*18*). Hepatocytes contained a single round nucleus with a prominent single nucleolus. The cytoplasm gave variable amounts of basophilic staining frequently concentrated at the cell center immediately around the nucleus. Hepatocyte margins were visible as a thin line contrasting with the large clear staining areas in the cytoplasm between nuclei and plasma membranes (figs. 1, 2). Bile ducts were more commonly found at the porta hepatis and rarely at depths within the livers. However, small oval cells were frequently seen in the center of hepatic tubules (fig. 1) and were thought to be bile preductular lining cells (*19*). No lobular differences were encountered. By TEM, cells were uniform and contained a single round nucleus with a prominent nucleolus (fig. 3). Rough endoplasmic reticulum, mitochondria, and dense bodies occupied a perinuclear cuff (*20*) or were dispersed (fig. 3). From this perinuclear cuff, extensions coursed peripherally to the plasma membrane and divided large regions of the cytoplasm in which primarily glycogen and occasional lipid droplets were found.



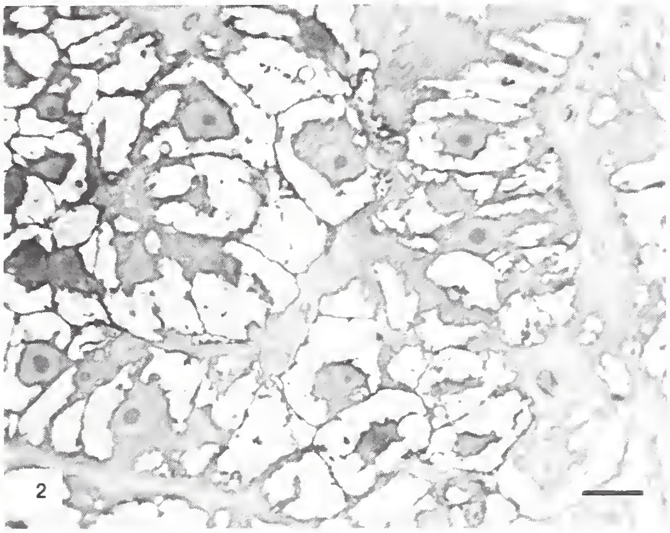


FIGURE 1.—Control liver showing small oval cells (O) between hepatocytes and sinusoids (S). H & E. Bar = 50 μm. × 240

FIGURE 2.—Control liver. *Note* single rounded nucleus with prominent nucleolus and perinuclear staining. Toluidine blue-stained Epon-Araldite section. Bar = 10 μm. × 1,100

The most striking feature of these cells was the abundant glycogen and the occasional large-to-medium-sized lipid droplets which were extensive in some control fish (fig. 3). No ultrastructural differences were seen in hepatocytes that would be indicative of lobular zonation. All the above morphological findings are similar to previous reports in control medaka (*4, 5*).

Age Observations

No striking changes were seen in control livers as a function of the age of the fish. Larval hatchlings and fish 3, 6, 9, and 12 months old showed similar architecture. The only apparent change may have been in the appearance of the bile ducts. These were always observed at the porta hepatis in all age groups but become more easily recognized in intrahepatic regions in older animals.

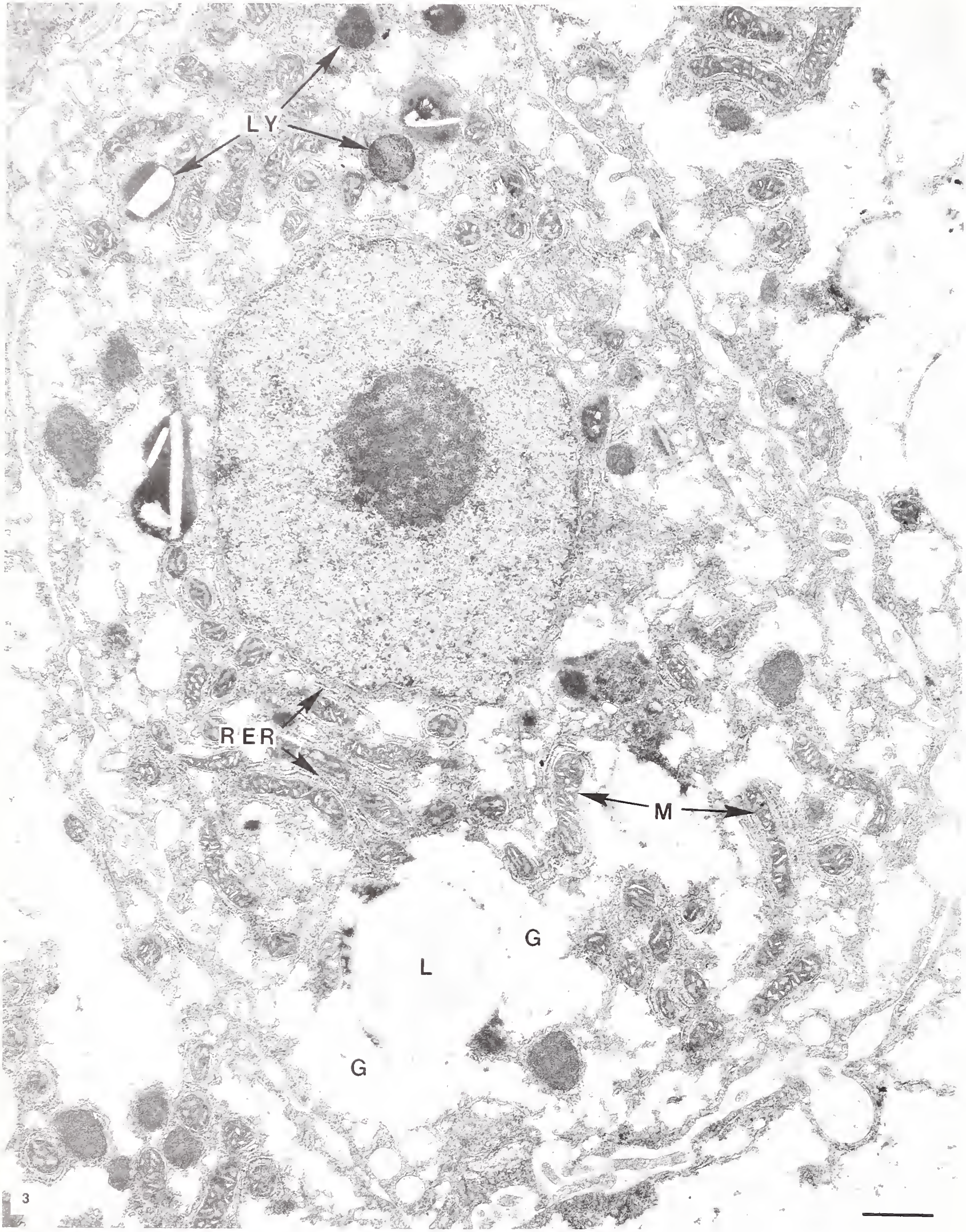
Carcinogen Treated

The H & E-stained paraffin sections from fish in the 66-day postexposure group (n=4) showed evidence of degeneration and necrosis (fig. 4). In general, hepatocytes showed rounded margins that suggested cell swelling. Degenerative lesions included: pigment-laden macrophages, intranuclear inclusions, granular eosinophilic cytoplasm, and occasional amorphous acidophilic masses. Cyst-like spaces (fig. 5) of variable size (from 1 to 2 cells in width to 1.5 mm wide) were seen in 2 of 4 animals. These appeared to arise through degeneration and possibly death and dissolution of hepatocytes. All fish showed foci of alteration characterized by areas of enhanced staining and disruption of normal architecture. Using the definition of Japanese workers (*5*), we believed these to be circular foci of increased staining. The liver from a single fish was grossly macronodular, and the lesion (fig. 6) appeared identical to hepatocholangioma described in small aquarium fish after exposure to various carcinogens (*1–5*). A nodular hepatocellular adenoma (*1*) was noted in the liver of another fish 66 days after exposure (fig. 7).

Livers from fish killed 116 days (n=8) after exposure to MAM acetate reflected a variable degree of return toward normal liver architecture. All livers had areas with no apparent changes from control, and 2 of 5 livers were completely absent of notable alteration. Of the remaining animals, the most prevalent observation was granuloma (in 3 of 5) in close proximity to cystlike spaces (fig. 8). No gross macronodular changes were seen in this group, but 2 of 5 fish showed small basophilic foci, and 1 of 5 showed a focus of acinar architecture partially separated from adjacent liver by connective tissue. Areas of bile duct proliferation with mitotic figures and variation in lining epithelium, from cuboidal to tall columnar, were seen in 1 fish.

Our findings in the 116-day postexposure group were substantiated and extended by HRLM. The cellular alterations seen were characterized by rearrangement of dark-staining material, some of which was pushed toward the periphery of the cell with an apparent decrease in clear, unstained areas (fig. 9). Nuclei and nucleoli were irregular and appeared enlarged. Rare nuclear pseudoinclusions were seen. Mitotic figures, rarely seen in hepatocytes, were more commonly observed in bile ducts. In addition, lesions characteristic of degeneration and chronic inflammation were noted. Large cystlike spaces were seen that were apparently devoid of hepatocytes. Some of these areas were clear and lacked stainable material, whereas others contained low density material, gray in color that was apparently surrounded by attenuated portions of cells. Frequently, the vacated region was the site of granulomas containing small cells with high nuclear-to-cytoplasmic ratios, pale staining, and no glycogen. These may represent macrophages. Dark-staining particulate material was seen in some hepatocytes, though others showed large vacuoles with cleftlike spaces (fig. 9).

Observations under TEM showed ultrastructural changes in livers of these fish 116 days after exposure to MAM acetate. Hepatocytes were recognized by their large clear regions in the cytoplasm but cellular organelles were altered (fig. 10). Mitochondria appeared as "dumbbell" shapes with highly attenuated centers. Numerous dense bodies indicative of lysosomal interaction and autophagic vacuoles were seen. Most apparent was an increase in the thickness of the rough endoplasmic reticulum extending from the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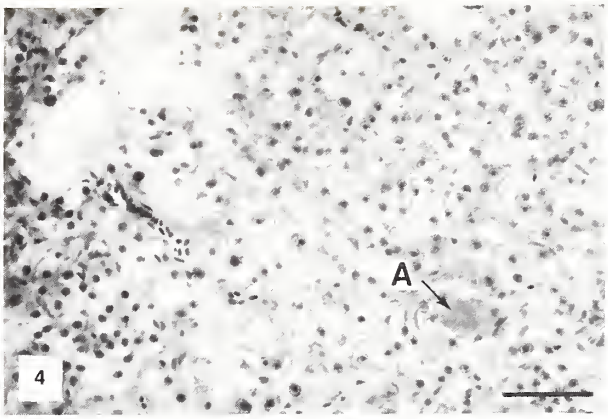
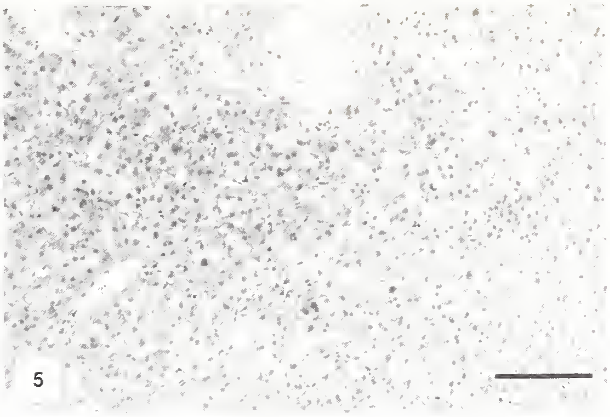
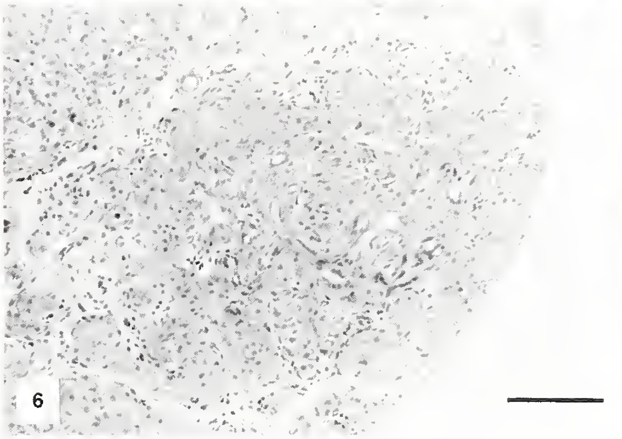






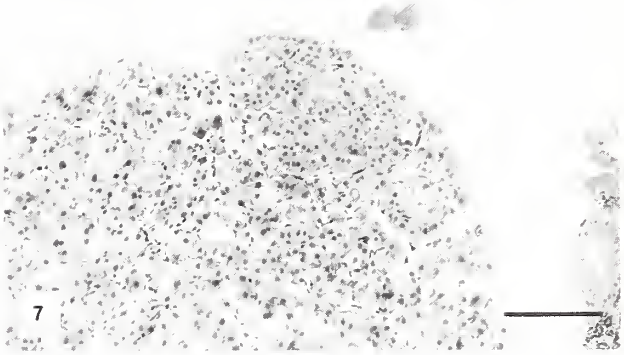


FIGURE 4.—Degenerative lesions and necrosis at 66 days postexposure to 0.5 ppm MAM. Cell swelling and amorphous acidophilic mass (A) are seen. H & E. Bar=50 μm. × 240

FIGURE 5.—Cyst-like space in liver of fish from 66-day exposure group. H & E. Bar=100 μm. × 120

FIGURE 6.—Small hepatocholangioma in 66-day post exposure liver. H & E. Bar=100 μm. × 120

FIGURE 7.—Small hepatocellular adenoma near liver surface 66 days after exposure. H & E. Bar=100 μm. × 120

perinuclear region out into the remainder of the cytoplasm thus causing an apparent decrease in the glycogen (fig. 10).

Cystlike spaces were lined with cells containing abundant intermediate filaments (10-nm diameter) and basement membrane-like material (fig. 11). These cells also contained numerous vacuoles of uniform size. Many of the latter appeared to contain basement membrane-like material. Desmosomes were prominent (fig. 12), and the filaments associated with these revealed continuity with bundles of intermediate filaments. Other cells, probably macrophages, were seen that contained numerous lysosomes and heterophagic vacuoles.

Granulomas noted in LM and HRLM contained uniform cells that existed as clumps of abundant cells with round contours. These had cytoplasm of low electron density that contained numerous lysosomes and heterophagic vacuoles (fig. 13). The peripheral portions of the granulomas contained cells with cytological features consistent with hepatocytes.

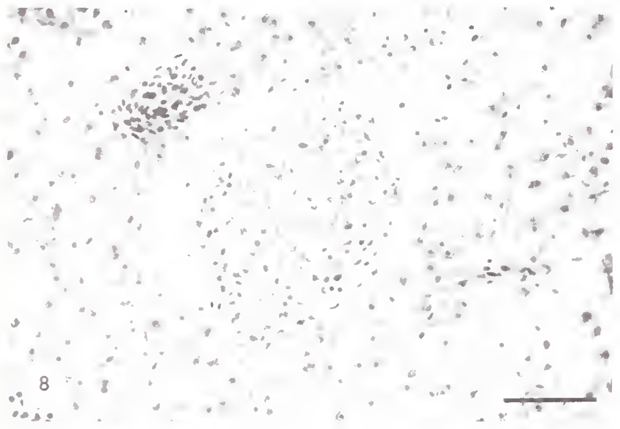
Quantitative Observations

Morphometric evaluation of H & E-stained paraffin sections of livers from various ages of medaka did not show any significant differences in V_v of hepatocytes, bile ducts, blood spaces, or other components. Therefore, all animals in the age study were lumped into 1 group, and the data are presented as controls in table 1.

Significant differences were seen after LM evaluation of MAM-treated fish (table 1). At 66 days after exposure, livers showed significant decreases in V_v of hepatocytes and blood space and significant increases in V_v of bile ducts and other components. After 116 days, the differences in hepatocytes and bile ducts had disappeared, which indicated a return toward more normal liver parameters.

←

FIGURE 3.—Control hepatocytes under TEM. Uranyl acetate and lead citrate stain. A single nucleus with a prominent nucleolus is shown. Rough endoplasmic reticulum (RER), mitochondria (M), lysosomes (LY) with and without cleft-like spaces and lipid droplets (L) are seen. Glycogen (G) deposits occupy large spaces. Bar=1 μm. × 14,000



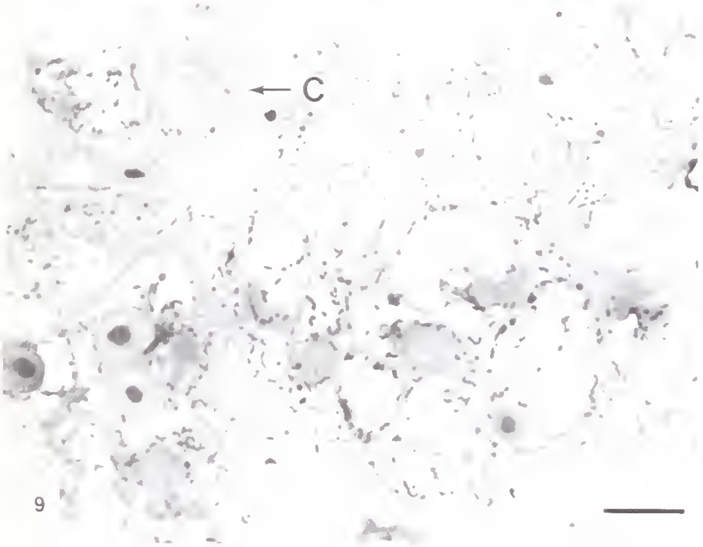


FIGURE 8.—Granuloma adjacent to cystlike space in liver of a fish 116 days after exposure. H & E. Bar = 100 μm. × 120

FIGURE 9.—Hepatocytes in fish 116 days after exposure showing dark-staining material which has been displaced toward the cell periphery. Cleftlike spaces (C) are seen in a macrophage. Toluidine blue-stained Epon-Araldite section. Bar = 10 μm. × 1,100

TABLE 2.—*Effect of MAM acetate on medaka hepatocytes*

Cellular parameter, V_v[a]	Control[b]	Treated[b]
Nuclei	5.4 ± 2.0	9.1 ± 1.0^{c}
Nucleoli	0.4 ± 0.5	0.8 ± 0.8^{d}
Nonstained cytoplasm[e]	16.3 ± 4.4	20.5 ± 6.0
Toluidine blue-stained cytoplasm	40.3 ± 10.8	44.7 ± 7.1
Plasma membrane and stained cell periphery	14.7 ± 3.5	21.7 ± 2.8^{f}
Lipid droplets	23.3 ± 16.5	3.9 ± 4.0^{d}
Hepatocytes[g]	83.5 ± 8.1	87.5 ± 2.5

[a] *See* footnote *a*, table 1.

[b] *See* footnote *b*, table 1.

[c] $P<0.005$.

[d] $P<0.05$.

[e] Nonstained cytoplasmic areas = clear areas which by TEM are occupied by glycogen particles.

[f] $P<0.01$.

[g] Percent of total liver occupied by hepatocytes, all other values = percent of hepatocyte volume.

However, blood space V_v remained significantly below control, and V_v of other components, though lower than in the 66-day postexposure group, was still significantly increased over control values.

At the cellular level, changes in hepatocytes were seen in the 116-day postexposure group (tables 2, 3), evaluated by HRLM. The percentage of volume of the hepatocytes taken up by various cellular components is shown in table 2. Significant increases were seen in treated livers with respect to nuclei, nucleoli, plasma membrane, and peripheral dark-staining cytoplasm. The V_v of lipid droplets was significantly decreased in treated animals. No changes in nonstained cytoplasm (glycogen) or perinuclear staining material were observed. An increase in nuclei and nucleolar V_v is indicative of increases in nuclear-to-cytoplasmic ratios in treated hepatocytes, whereas increased peripheral staining may be due to increases in peripheral endoplasmic reticulum.

TABLE 1.—*Effect of MAM acetate on medaka liver*

Parameters, V_v[a]	Control[b]	Treated[b]	
		66 Days	116 Days
Hepatocytes	88.5 ± 1.0	80.1 ± 3.4^{c}	86.6 ± 2.8
Bile ducts	0.057 ± 0.082	1.00 ± 0.53^{d}	0.40 ± 0.40
Blood space[e]	11.1 ± 1.0	9.3 ± 1.3^{d}	8.4 ± 1.1^{f}
Other[g]	0.31 ± 0.22	9.6 ± 3.5^{f}	4.6 ± 2.9^{d}

[a] V_v = dimensionless ratio expressed as percent of total.

[b] For No. of animals/group and details of morphometric examination, *see* "Materials and Methods." Values are means ± SD.

[c] $P<0.01$.

[d] $P<0.05$.

[e] Sinusoids as well as all blood spaces are included.

[f] $P<0.005$.

[g] Other structures include connective tissue and melanomacrophage centers.

TABLE 3.—*Average cell data in control and MAM-treated hepatocytes*

Parameter	Control[a]	Treated[a]
Hepatocyte volume	$3{,}480 \pm 2{,}900\ \mu m^3$	$814 \pm 197\ \mu m^3$
Nuclear volume	$134 \pm 36.5\ \mu m^3$	$73.0 \pm 14.8\ \mu m^{3b}$
Nucleolar volume	$2.0 \pm 1.4\ \mu m^3$	$1.4 \pm 0.4\ \mu m^3$
Hepatocyte surface area	$1{,}937 \pm 1{,}447\ \mu m^2$	$583 \pm 116\ \mu m^2$
N_v hepatocytes, cm^{-3}	$3.79 \pm 1.86 \times 10^{8}$	$11.5 \pm 2.94 \times 10^{8c}$

[a] *See* footnote *b*, table 1.

[b] $P<0.01$.

[c] $P<0.005$.

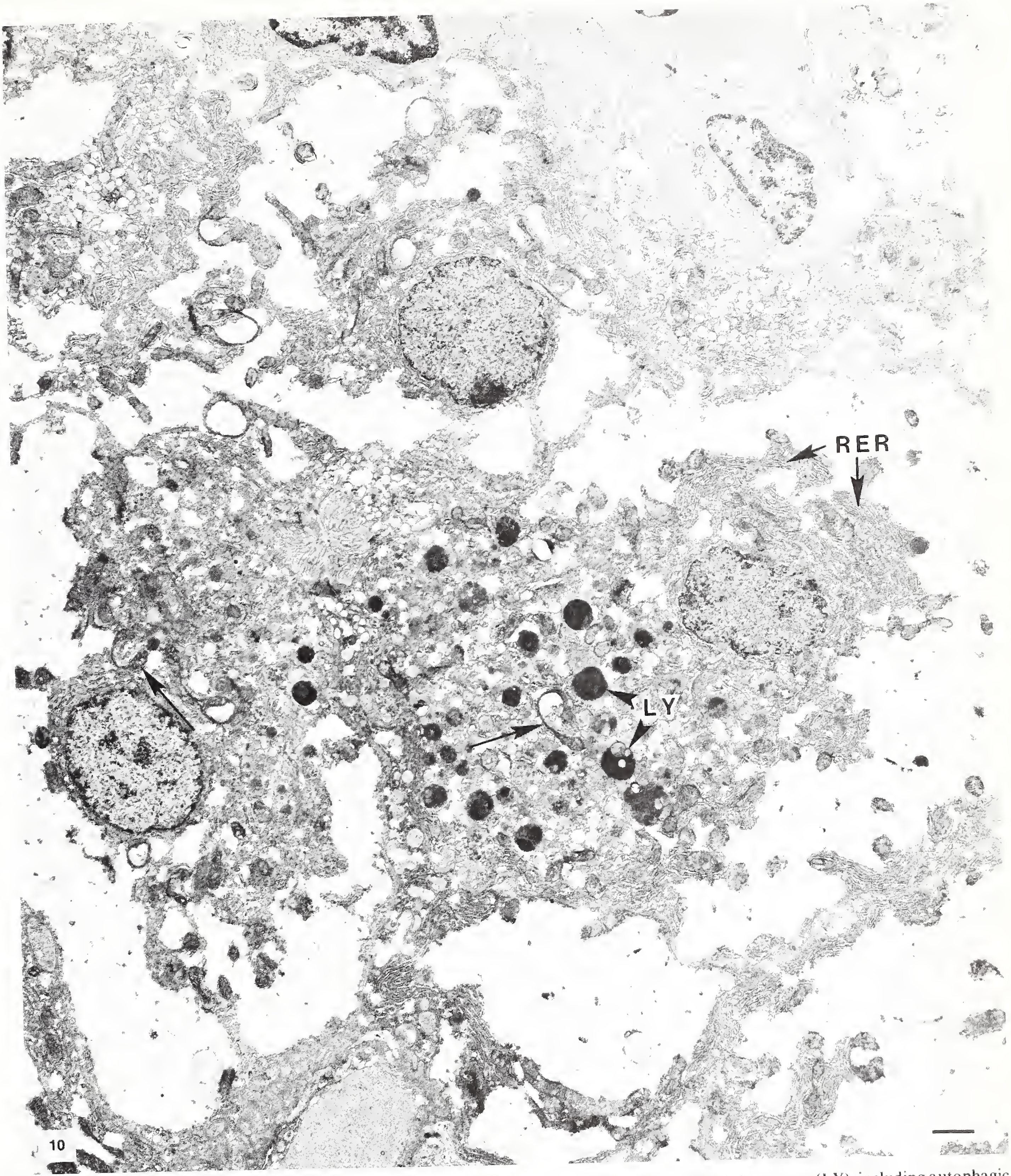


FIGURE 10.—Hepatocytes after 116 days exposure show dumbbell-shaped mitochondria (*arrows*), lysosomes (LY), including autophagic vacuoles and increased thickness of perinuclear zone containing well-developed rough endoplasmic reticulum (RER). Large clear zones contained glycogen. Uranyl acetate and lead citrate. Bar = 1 μm. × 7,280

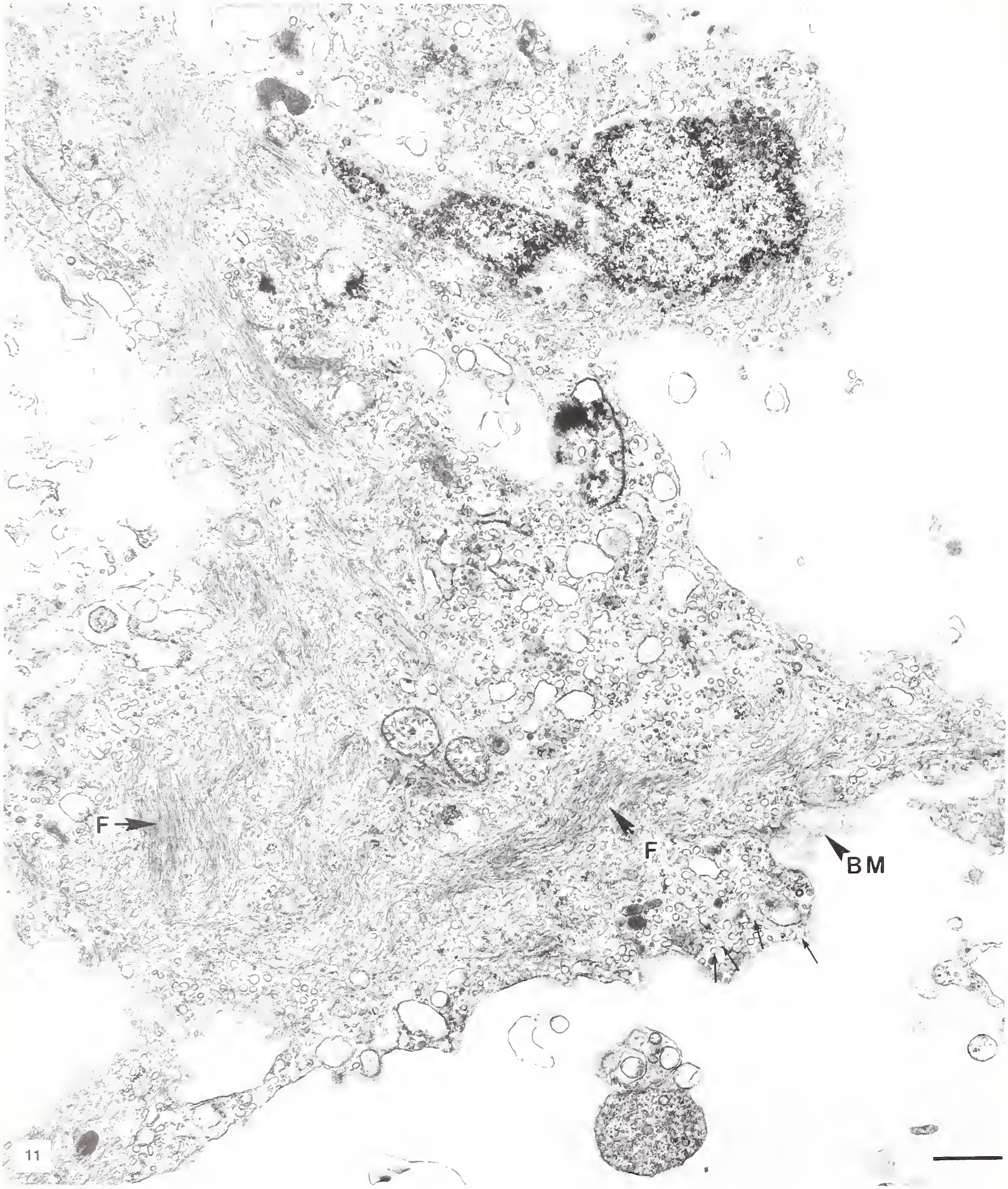


FIGURE 11.—Empty fat-storing cell at periphery of cystlike space (116 days postexposure) showing numerous intermediate filaments (F), abundant basement membrane-like material (BM), and numerous vacuoles of uniform size. Basement membrane-like material was seen in vacuoles (*arrows*). Uranyl acetate and lead citrate. Bar = 1 μm. × 7,280

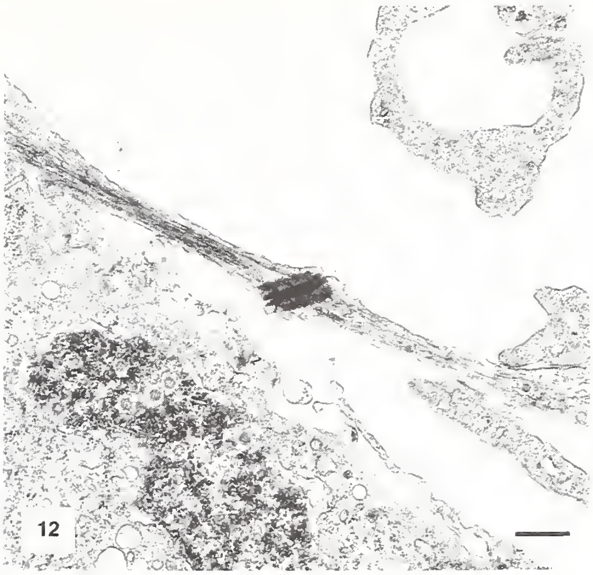


FIGURE 12.—Higher magnification from peripheral portion of cell shown in figure 11 reveals continuity of intermediate filaments with desmosome. Uranyl acetate and lead citrate. Bar=0.5 μm. × 15,000

Average cell data calculated from morphometric measurements (table 3) show trends toward smaller cell volumes and surface areas. Although the differences in means between treated fish and controls for these 2 parameters are large, they are not significantly different when tested by analysis of variance because of the large variation in cell size between different animals in the control group. Average nuclear volume was significantly decreased in treated animals. The most striking change was the number of hepatocytes per cubic centimeter. The MAM-treated liver contained approximately three times as many cells as controls in the same volume.

DISCUSSION

Significant differences were produced by MAM acetate in livers of medakas 66 days after exposure. Decreased V_v of hepatocytes and blood space and increased V_v of bile duct and other components were seen. However, whole liver parameters returned toward control values at 116 days postexposure. Cellular parameters (as studied in the 116-day group only) indicated significant alterations in hepatocytes. Increases in nuclear-to-cytoplasmic ratio and in the volume compartment for dark-staining cytoplasmic material were seen with a 300% increase in the number of hepatocytes per cubic centimeter. These objective quantitative evaluations confirm and extend qualitative observations both in this and earlier work (*5*). Areas of cellular alteration seen at 66 and 116 days after exposure included macronodules, basophilic foci, and increased mitoses as well as alteration of liver architecture. These microscopic observations are similar to those reported by the Japanese workers (*5*). However, the incidence of tumors in our studies was lower. Aoki and Matsudaira (*5*) found that treatment of 1-year-old medakas with 0.5 ppm MAM acetate for 10 days produced tumors including trabecular hepatoma and cholangioma in 100 and 83% of the livers 60 and 90 days after exposure, respectively. In the present study, 1 of 5 fish showed macronodular change at 66 days afterward. However, if foci and macronodular changes are grouped together, we saw 100% change at 66 days and 60% at 116 days.

The existence of large cystlike spaces in livers at 66 and 116 days and the development of granulomas adjacent to the regions in the latter groups were interpreted as evidence of degeneration including necrosis and chronic inflammation. Previous work in medakas has not shown this degenerative and chronic inflammatory type of response (*5*), although similar responses were seen in zebra fish after exposure to diethylnitrosamine (*1*). We have been impressed by the similarity of the cystlike spaces in this study and spongiosis hepatis (*21*) described in rat liver after administration of *N*-nitrosomorpholine. Both involve perisinusoidal liver cells. Based on comparison with ultrastructural descriptions in various teleosts (*22–24*), we believe the filament-rich cells to be empty fat-storing cells (*23*). Interestingly, fat-storing cells are abundant in the spongiosis hepatis of rat liver (*21*).

The return of more normal appearing liver structure between 66 and 116 days postexposure may be indicative of repair and resolution of the degenerative lesion (*1*).

The tremendous increase in the number of hepatocytes per cubic centimeter of liver with a significant change in the nuclear-to-cytoplasmic ratio is indicative of altered hepatocyte populations. Whether these alterations are hyperplastic and appear in response to repair to toxic effect or whether they are truly neoplastic cannot be determined at this time. Kinetic studies in which tritiated thymidine is used indicate enhanced mitotic activity in *Oryzias* after exposure to MAM acetate (*25*).

We must point out that in this study all areas in the liver were analyzed in each of the groups. Therefore, if foci of alterations are small, any change measured in these altered areas would be expected to be diluted with data from more normal areas resulting in lower estimations of the total liver changes.

Investigators (*26*) have used morphometric techniques to assess morphological alteration only sparingly in carcinogenesis research. We believe this is a powerful technique providing objective data which can be used in statistical testing between groups or in the determination of a dose–response based on morphological criteria. It is our intention to continue to use these techniques in extended ultrastructural studies to increase our knowledge of control cellular structure and compare these with morphometric data from carcinogen-exposed fish. In this manner, we hope to define more clearly and measure more accurately the effects of exposure on the liver cell populations and on their organelle components.

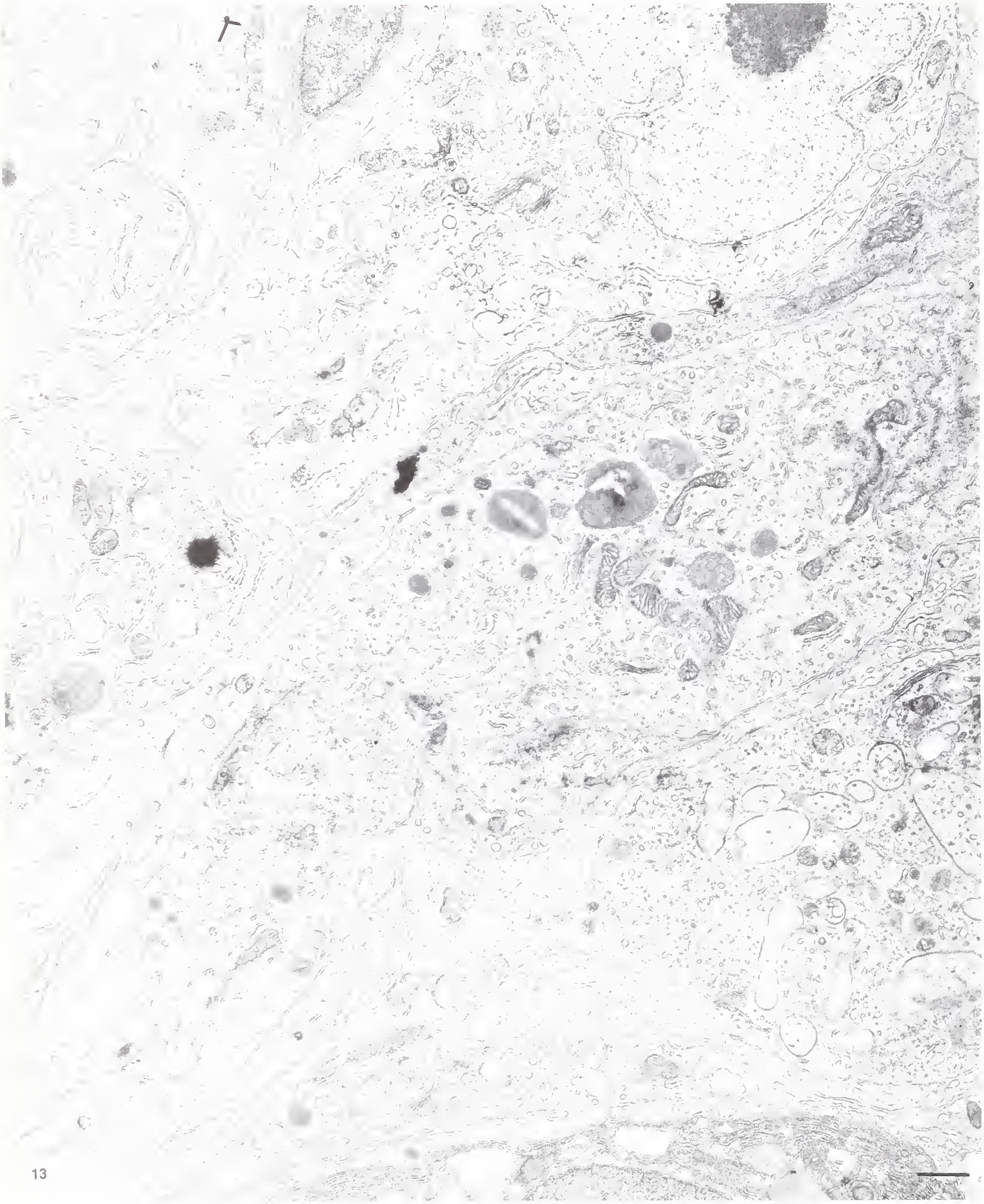


FIGURE 13.—Macrophages in granuloma (116 days postexposure) show numerous lysosomes and heterophagic vacuoles. Uranyl acetate and lead citrate. Bar = 1 μm. × 9,570

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DISCUSSION

B. Trump: I have two questions. What happened to the liver weight in the treated animal?

D. E. Hinton: These are very small fish, and we did not take liver weights this first time.

Trump: The comparison of the volume and numbers suggest it must have gone up because the volume should have been multiplied by eight, roughly, with what you saw in the numbers.

The other question relates to your previous talk. In the quantitation of the electron microscopy level of the smooth or rough endoplasmic reticulum, but especially the smooth (as you know, in the rodent or in the human, you cannot really visualize that as long as glycogen is present), what happens to the endoplasmic reticulum? Would that not be a more appropriate base line for comparing the hydrocarbon-treated fish? Has that been done? Have you done that? If all you do is deplete the glycogen, e.g., by starvation or by epinephrine, it makes a big difference in the visualization of the endoplasmic reticulum of the rat.

Hinton: That is right. In the rat, the first component to change is the rough reticulum initially, and then the vast component changing in the total endoplasmic reticulum is due to the smooth. In the fish, you have a nice system for studying smooth endoplasmic reticulum because it is hard to stain the glycogen unless you use primary osmium fixation or reduced osmium or process rapidly in the cold, and/or go through other special techniques. Usually you lose the glycogen and get a washed-out appearance, and that makes it much easier to see the membrane.

Trump: However, Dr. Keith Porter showed this long before we knew how to stain glycogen with alkaline lead. It was washed out there, too.

Hinton: It is much easier to see. I think the fish is going to be handy for these types of studies.

Pseudoneoplasms in Ectothermic Animals[1, 2]

John C. Harshbarger[3]

ABSTRACT—Gross and cytologic similarities between certain non-neoplastic conditions and neoplasms in lower animals including fish, amphibians, and reptiles have invited misinterpretations and contested interpretations. Major categories of pseudoneoplasms, illustrated by specific examples from material accessioned into the Registry of Tumors in Lower Animals, include infections by foreign organisms which resemble host cells (algal protothecosis and amebic pseudotumors); unusual normal conditions [giant islets of endocrine pancreas (Brockmann bodies) in liver, atypical sites of hematopoietic tissue]; nonparasitic hyperplasia (goiter, ectopic thyroid, erythroblastic proliferation suggestive of pernicious anemia, adenofibrosis); parasite-induced hyperplasia (trematode-induced fibrosis, ciliate-induced monocytic leukocytosis, trematode-induced melanosis, glochidiosis); dysmorphogenesis (teratoid anomalies); virus-induced hypertrophy (lymphocystis); and reactive lesions (metaplasia, regeneration, inflammation). — Natl Cancer Inst Monogr 65: 251–273, 1984.

Non-neoplastic conditions, with features resembling neoplasms, may be misdiagnosed as neoplasms, and, to a lesser extent, the reverse is also true. Common difficulties include correct identification of the origin and type of cells involved; the distinguishing of reactive lesions from neoplasms; differentiation of borderline conditions, such as hyperplasia or developmental anomalies, from neoplasia; and separation of neoplasia from other disease conditions that occur simultaneously in the same lesion. Investigators may reduce errors and uncertainties by increased familiarity with the subject, but contention about the neoplastic versus non-neoplastic nature of a condition may still be protracted and difficult to resolve.

The source of the difficulty is the lack of any known universal morphological or biochemical trait that is pathognomonic for neoplasia. Neoplasms are clones of cells with vertically transmitted abnormalities manifested by varying degrees of aberrant behavior, including autonomy from host control, progressive proliferation, cellular anaplasia, and tissue disorganization. Invasion of adjacent tissues and metastasis to remote locations indicate malignancy. Diagnosis is an interpretation of the sum of cytological observations related to the natural history of similar appearing cell populations previously encountered. A multiplicity of contributing factors, including cell and tissue types, host species, nutritional state, hormonal state, oncogenes activated, initiators, promoters, and temperature, allow an enormous number of tumor and tumor-like permutations. As a result, enigmas abound to the extent that proliferation of a specific cell type in different species or even in different organs within the same species may be neoplastic in one case and hyperplastic (reversible proliferations of normal cells) in another (*1*). Thus it is not surprising that compensatory or regenerative hyperplasia, parasites, host responses to foreign organisms, toxicoses, teratoses, or even normal structures have been misinterpreted as neoplasms and vice versa.

PROTOTHECOSIS DIFFERENTIATED FROM CHONDROSARCOMA

Embedded and sectioned tissue was contributed to the Registry (RTLA 1107) by C. G. Crispens, Jr., from a primary and a recurrent lesion that developed in the ventrolateral anterior trunk area of a 3½-year-old, male, captive corn snake, *Elaphe guttata guttata*. The primary lesion developed within a year to an oval surface growth 2.5 cm long. Following surgical removal of the primary, regrowth was rapid. Removal of the recurrent lesion at about 3 weeks was followed within hours by hemorrhage and death.

The histological appearance of both surgical specimens was similar. The tissues consisted of a large mass of thick-walled, unpolarized cells, each with a small nucleus containing a large nucleolus and a large, clear cytoplasm (fig. 1). Superficially, the cells resembled mature chondrocytes and suggested a diagnosis of chondrosarcoma. However, comparison with a metastatic, culturable chondrosarcoma from a corn snake (*2*) previously contributed to the Registry by J. D. Small (RTLA 465), showed distinct differences (fig. 2). The chondrosarcoma had larger nuclei with smaller nucleoli; cell walls were thin and PAS-negative versus thick, strongly PAS-positive cell walls for the unknown; and an extracellular matrix had been produced by the chondrosarcoma versus no matrix for the unknown. The chondrosarcoma had areas of precartilage, sometimes with intercartilaginous mineral deposits confirmed by alizarine red S and Von Kossa stain (fig. 3), whereas the unknown had no corresponding areas. Cells of the

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ABBREVIATIONS: RTLA = Registry of Tumors in Lower Animals (Smithsonian Institution, Washington, D.C.); PAS = periodic acid-Schiff.

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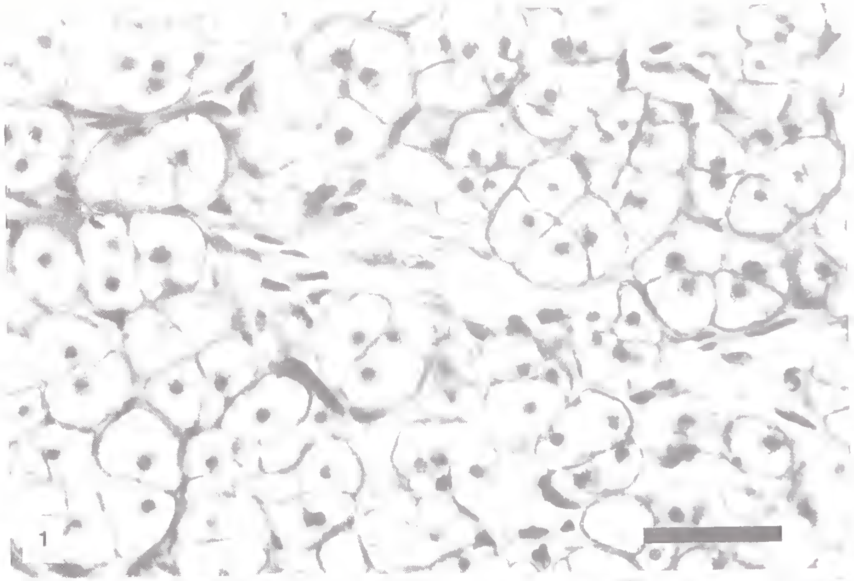
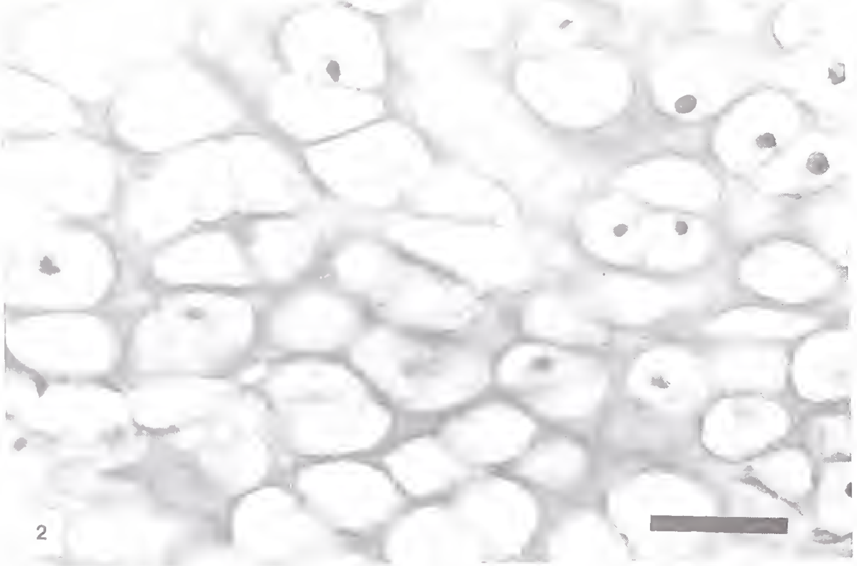
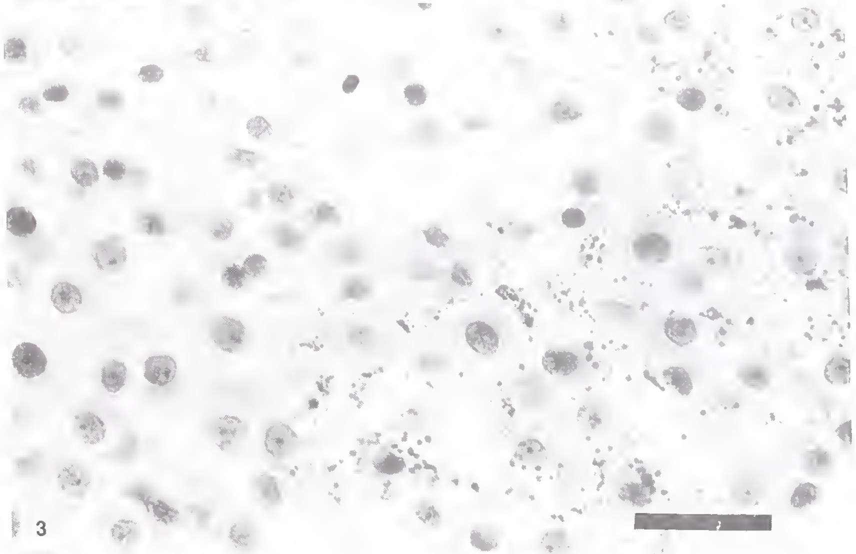
[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Supported by Public Health Service contract CP26000 from the Division of Cancer Cause and Prevention, National Cancer Institute.

[3] Registry of Tumors in Lower Animals, National Museum of Natural History, Smithsonian Institution, Washington, D.C. 20560.







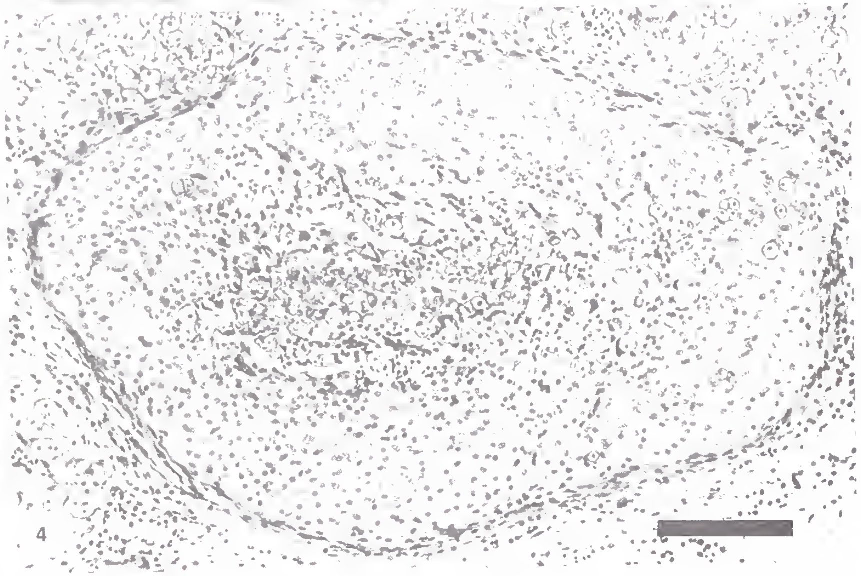


FIGURE 1.—Protothecosis in a corn snake (RTLA 1107). Algal cells have thick cell walls, clear cytoplasm, small nuclei with large nucleoli, and occur in tetrads. No intercellular matrix. Bar = 30 μm.

FIGURE 2.—Chondrosarcoma in a corn snake (RTLA 465). Mature chondrocytes resemble algae in figure 1 as large cells with clear cytoplasm, but differ in thinner cell walls, larger nuclei with smaller nucleoli, and presence of intercellular matrix. Bar = 30 μm.

FIGURE 3.—Chondrosarcoma (RTLA 465). Immature chondrocytes have thin cell walls, light cytoplasm, large nuclei, small nucleoli, no appreciable intercellular matrix, but intercellular calcium granules are present. Bar = 30 μm.

FIGURE 4.—Protothecosis (RTLA 1107). Granuloma or focal chronic inflammatory reaction. Center contains necrotic algae and leukocytes. Intermediate layer consists of epithelioid monocytes with a scattering of viable algae. Periphery is a thin fibrous capsule. Algae predominate extracapsular field. Bar = 30 μm.

unknown, in contrast, tended to form tetrads and, in some areas, elicited response by inflammatory cells (fig. 4).

These differences ruled out chondrosarcoma. The inflammatory reaction to the cells suggested a foreign origin, and the thick PAS-positive cell walls suggested a plant origin, which, with other morphological and behavioral features, was consistent with *Prototheca* sp., thought to be achlorophyllous mutants of the green alga, *Chlorella* sp. Such algal infections have been reported from cattle, dogs, deer, and humans (*3*), but this is the only case of protothecosis ever reported from a reptile (*4*).

AMEBIC PSEUDOTUMOR VERSUS X-CELL PAPILLOMAS AND X-CELL ADENOMAS

A Dover sole, *Microstomus pacificus,* from Departure Bay, Naniamo, British Columbia, was received from N. P. Boyce (RTLA 824) with a raised, 2.0 × 2.5 × 0.8-cm deep verrucal growth just caudal to the dorsal operculum on the eyed-side (fig. 5). On transverse cut, the lesion consisted of adjacent pegs of tissue which extended perpendicularly into the outer layer of dermis (fig. 6). Microscopically, surface layers of the covering epidermis appeared normal but pegs extending from the basal region contained scattered large, amorphous cells with coarse, granular acidophilic cytoplasm, large finely granular nuclei, and distinct nucleoli (fig. 7). Deeper large cells were densely packed but had undergone transition into vesicular and loosely granular moribund or dead ghosts except that some of those adjacent to radiating blood vessels maintained the viable appearance of those near the surface (fig. 8). High magnification revealed a network of cells with long cytoplasmic processes enveloping the large cells (fig. 9). A vigorous inflammatory reaction extended from the base of the lesion through the dense layer of the dermis, the subcutaneous tissue, and into skeletal muscle.

Similar large cells have been seen in skin lesions from 21 fish species of the orders Pleuronectiformes and Perciformes and in pseudobranch lesions from 2 species of order Gadiformes. They resemble no other cells ever described from these fish, and their cryptic origin has been emphasized by a reference to them as X-cells (*5*). Electron microscopy, flow microfluorometric nuclear DNA analyses, isoenzyme studies, and imprints of Pacific cod, *Gadus macrocephalus,* pseudobranch X-cell lesions, stained by Wright's, Feulgen, and bisbenzimidazole DNA-binding fluorescent dye, have revealed a good deal about their nature, including the probability that X-cells are not fish cells (*6, 7*). In synopsis, X-cells contained one-quarter to one-third as much chromatin as adjacent envelope cells and other fish cells that resulted in minute mitotic figures difficult for one to recognize in tissue section. Nuclear mitoses, which often occurred without cytoplasmic division, resulted in up to 60 synchronized plasmodial divisions per cell. X-Cells autofluoresced but fish cells did not. X-Cells and fish cells sorted by flow microfluorometry gave 2 different peaks. Cod tissue containing X-cells had 4–6 isoenzymes that were absent in any cod tissue that did not contain X-cells. The fine structure of X-cells from cod pseudobranch lesions was identical to that from flounder skin lesions (*5*). It was concluded (*7*) that X-cells are some type of protistan, having ameboid characteristics in host fish and possibly belonging to the Hartmanellidae.

This large group of protozoan lesions in three orders of marine fish was mistaken for various neoplasms for nearly three-quarters of a century despite the presence of a unique cryptic X-cell type, higher prevalence in younger than in older fish, underlying inflammation and sloughing of some lesions followed by healing, inconsistencies in attempted association with pollution, and other problematic features. Primarily, this error seems to have been due to the ability of the protistan to stimulate proliferation and envelopment by epidermal cells, thus protecting themselves from the host's immune system except when dead ameba in deeper areas presumably became unenveloped. It mimics neoplasia by producing a mass of unchallenged, fairly uniform cells in which the envelope cells appear to be part of the stroma.

NORMAL STRUCTURES DIFFERENTIATED FROM NEOPLASIA

Brockmann Bodies

A portion of liver from a spot, *Leiostomus xanthurus* (RTLA 2158; contributed by J. A. Couch) had a white nodule 2 mm in diameter. Based on the gross appearance (fig. 10), differential diagnoses included encysted parasite, neoplasm, and microbial granuloma. Microscopically, it was a huge, solitary, normal pancreatic islet (fig. 11). Although most bony fish have disseminated exocrine and endocrine pancreatic tissues within visceral organs and mesenteries, some have large, compact islets known as Brockmann bodies. According to Epple (*8*), Brockmann bodies were described by Stannius and Brockmann in 1846, more than 20 years before Langerhans discovered islet tissue first in rabbits, followed shortly in larval lampreys; he thought the tissue was exocrine. Brockmann bodies have been extracted for therapeutic and research purposes since the first decade of this century.

Hematopoiesis

A portion of liver from a tiger salamander, *Ambystoma tigrinum* (RTLA 2468; contributed by R. S. Anderson) had a smooth, convex nodule approximately 8 mm in diameter (fig. 12). Microscopically, it was an unusually large focus of normal subcapsular hematopoietic tissue (figs. 13, 14).

Material from the heart of an Atlantic sturgeon, *Acipenser oxyrhynchus* (RTLA 1118; contributed by T. R. Meyers) had what was described as watery lesions covering the entire surface. Microscopically, the surface was covered by a series of pericardial pouches, and, although the tissue was partially autolyzed, preservation was adequate in some places so that normal hematopoietic tissue within the pouch lumina could be distinguished (fig. 15).

PERNICIOUS ANEMIA VERSUS ACUTE GRANULOCYTIC LEUKEMIA

Six hematoxylin and eosin-stained microslides of tissues from 2 fingerling chinook salmon, *Oncorhynchus tshawytscha* (RTLA 1022, 1023; contributed by W. T. Yasutake), were representative of an epizootic disease in a

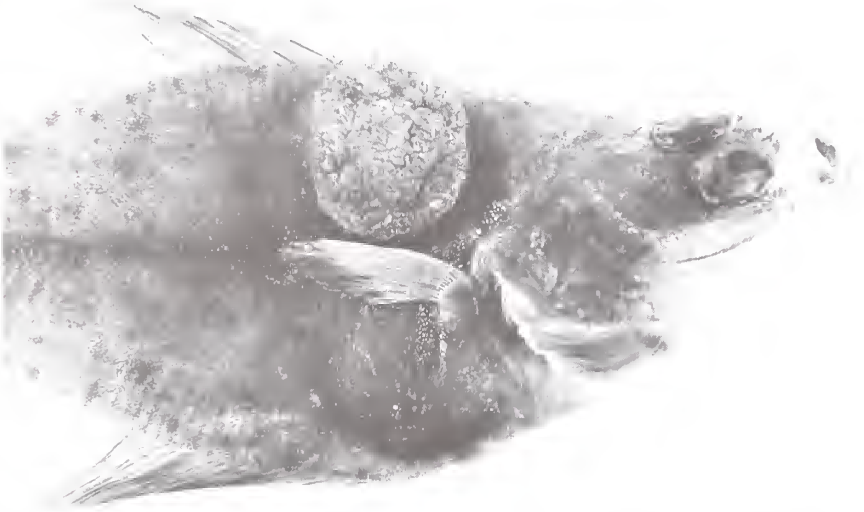


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FIGURE 5.—Amebic pseudotumor of Dover sole (RTLA 824). Verrucal lesion, 2×2.5×0.8 cm, above pectoral fin.

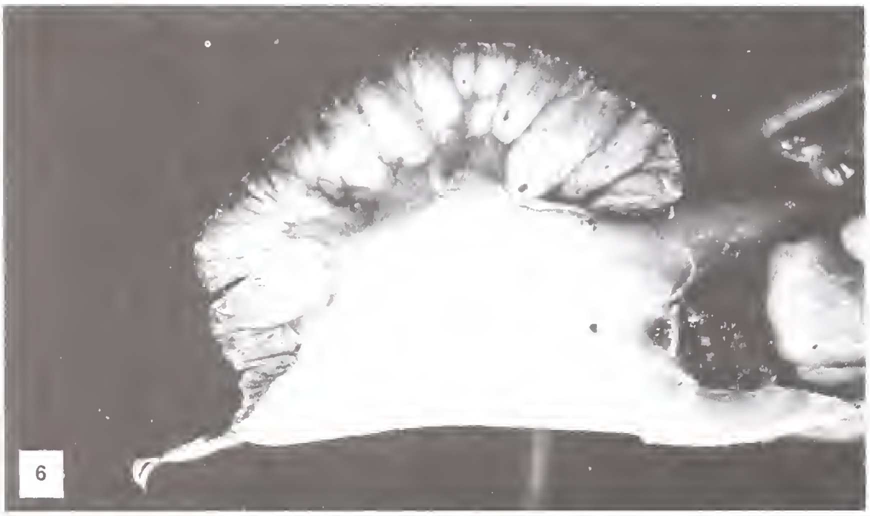

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FIGURE 6.—Cut surface of figure 5. Lesion (*top*) has multiple pegs resembling a papilloma. Pegs do not penetrate trunk muscle.

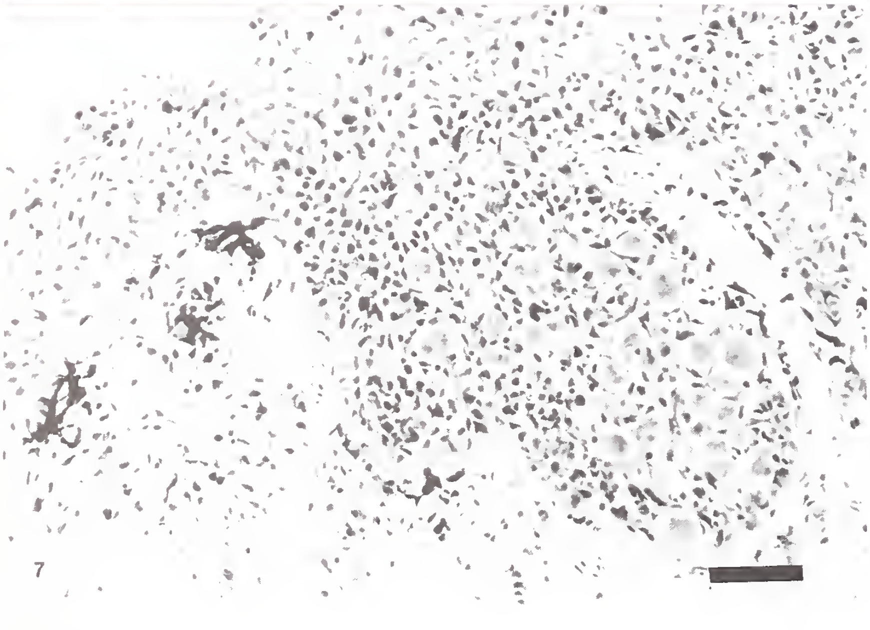

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FIGURE 7. — Amebic pseudotumor (RTLA 824). Peg of surface epidermis (*top center* to *lower right*) contains large amorphous cells with prominent nucleoli. Bar= 30 μm.

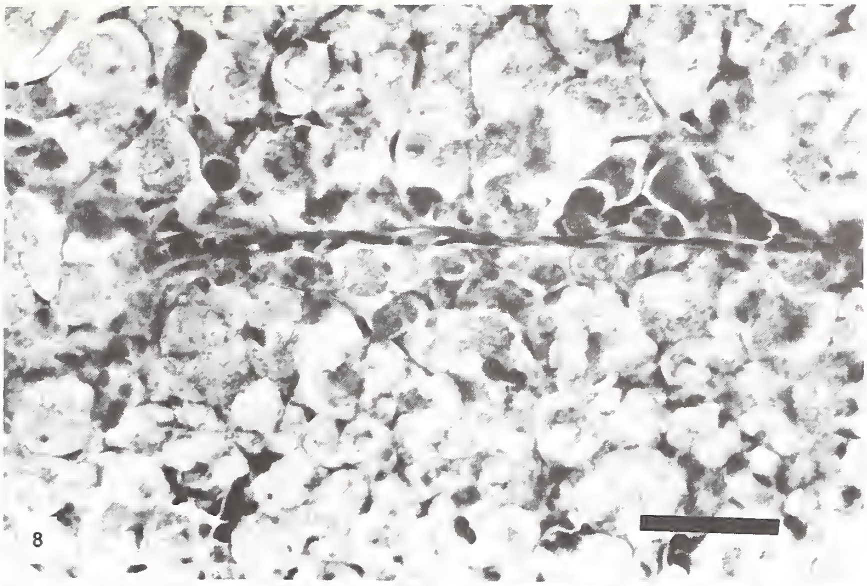

FIGURE 8. — Amebic pseudotumor (RTLA 824). Large amorphous cells are dying in deeper portions of lesion except along blood vessels (*center horizontal*). Bar = 30 μm.

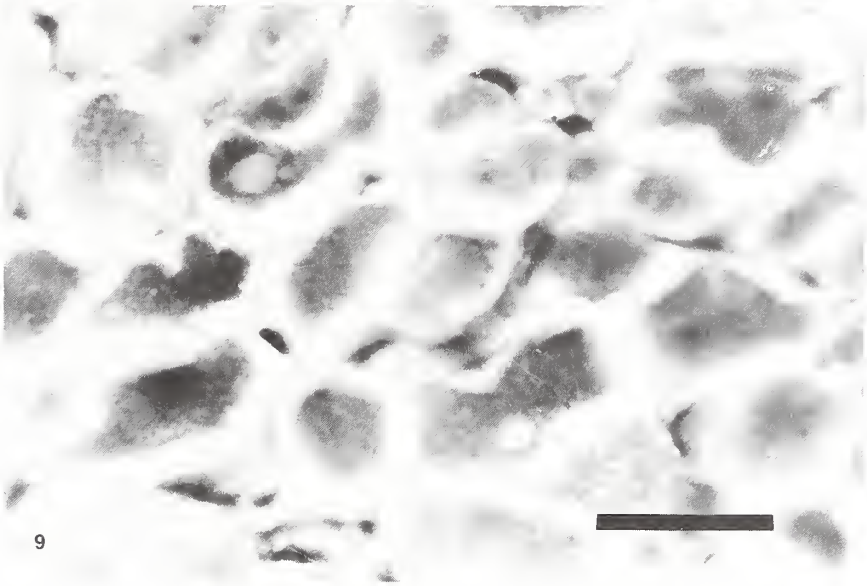

FIGURE 9. — Amebic pseudotumor (RTLA 824). Large amorphous cells are framed by network of flattened epithelial cells with long, thin cytoplasmic processes and occasional dense cell bodies. Bar = 15 μm.

hatchery. Gross signs shown by these 4-month-old salmon included moribundity, anemia, bilateral exophthalmia, ventral petechiae, and renal and hepatosplenomegaly. The incidence was originally estimated at 20%, but subsequent surveys indicated that only about 1% (6,000) of the 600,000 in the population were seriously afflicted. Microscopically (fig. 16), every tissue and organ including spleen, kidney, liver, pancreas, substantia propria of gut, visceral fat, and cardiac muscle contained immature, mitotically active cells which were also abundant in vessel lumina. These disseminated cells were blastlike with large, often deeply clefted, nuclei having abundant chromatin and 1 or 2 prominent nucleoli. Cytoplasm was abundant and contained a fine granulation. Granules were eosinophilic in a small portion of the cells, but no mature polymorphonuclear leukocytes were present, ruling out a leukemoid response to foreign antigens. As suggested by the contributor, the features were consistent with a diagnosis of leukemia, probably acute granulocytic in type. The possibility that an epizootic granulocytic leukemia could be mediated by a virus in a confined population of fish was intriguing, and a battery of collaborative studies was planned, including blood smears, tissue imprints, histopathology, electron microscopy, cell culture, reverse transcriptase, viral isolation, cell-free transmission, karyotype, hematology, and immunology. However, before these studies could be initiated the hatchery received an opinion from another source that the disease was pernicious anemia, even though in mammals, pernicious anemia usually occurs in older adults, and it has nutritional and physiological bases that are unlikely in this salmon fingerling population. Furthermore, the presence in these salmon of proliferating, arrested erythroblastic cells in tissues not known to have hematopoietic potential, such as

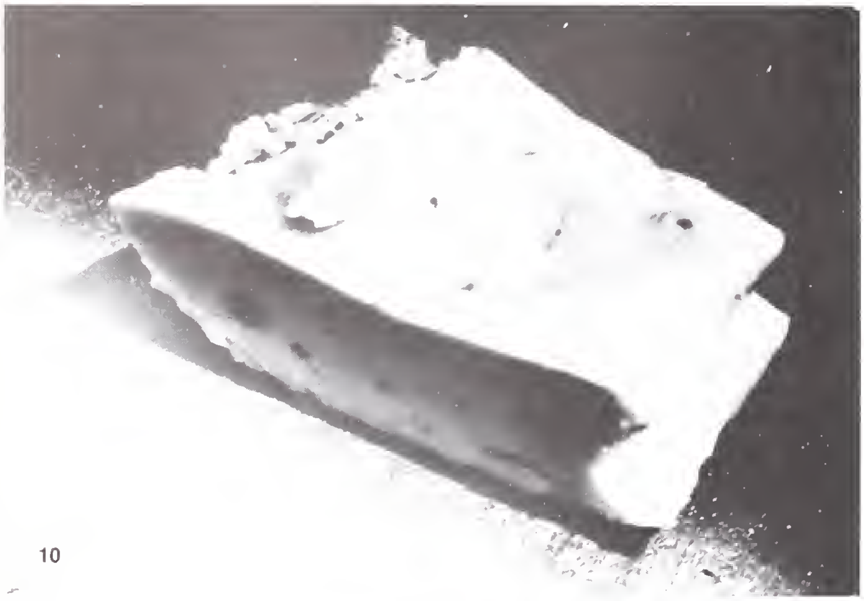

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FIGURE 10.—Brockmann body protruding from liver of a spot (RTLA 2158).

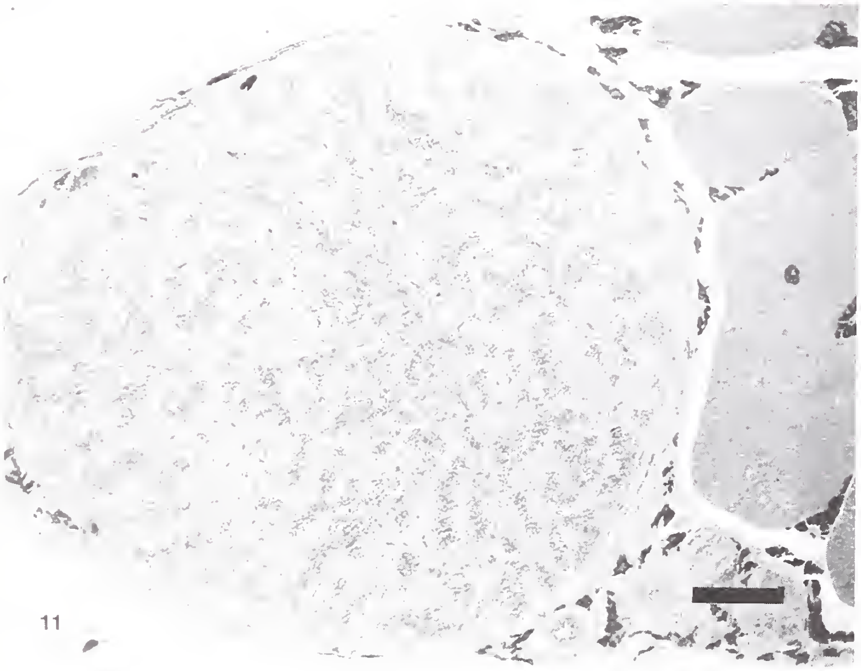

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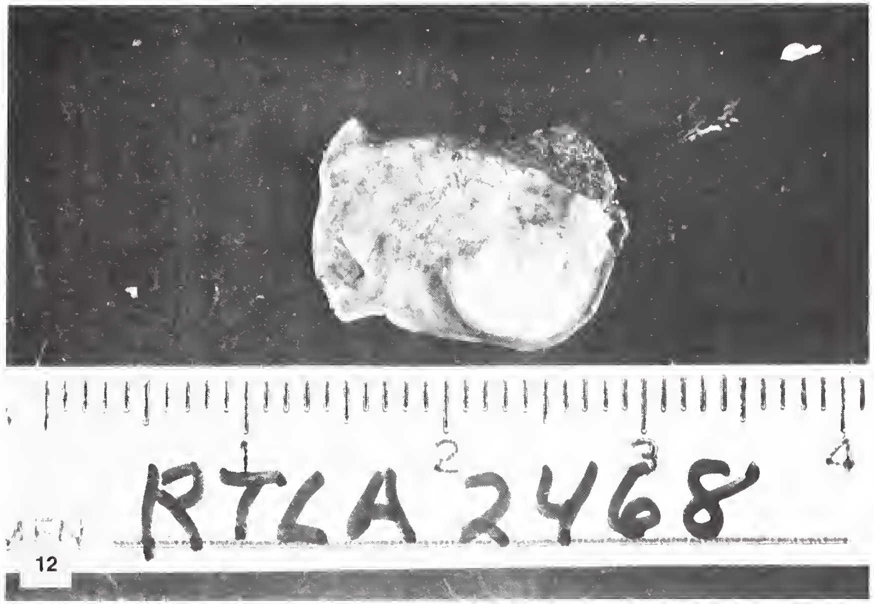
FIGURE 11.—Section of figure 10. Giant normal islet of endocrine pancreas or Brockmann body adjacent to normal liver (*right*). Dark tissue in liver exocrine pancreas. Bar= 200 μm.

pancreatic tissue, seems inexplicable. Unfortunately, before definitive studies could be undertaken, all the fish were released.

THYROID HYPERPLASIA DIFFERENTIATED FROM THYROID ADENOCARCINOMA

Thyroid tissue in bony fish is unencapsulated, is capable of widespread ectopic growth, is responsive to a wide range of goitrogens, and, when hyperplastic, may infiltrate and destroy normal tissues, such as bone, cartilage, and muscle. Distinguishing such hyperplastic behavior from neoplasia has been debated by scientists for a century as discussed by Hoover (*9*). An example of this difficulty is represented in a mass on a gill arch of a coho salmon, *Oncorhynchus kisutch,* believed to have been caught in Lake Erie (RTLA 2211; contributed by C. E. Smith). The mass, consisting of large expanses of well-differentiated thyroid follicles many of which contained colloid (fig. 17), was destroying cartilage (fig. 18), bone (fig. 19), and other tissues encountered in its expansion. Some areas of the mass were poorly differentiated (fig. 20), contained papillary ingrowths into the lumina (fig. 21), or had vascular emboli (fig. 22) which, in one instance, was growing from a vessel wall (fig. 22). This combination of features resembles neoplastic behavior, and one could be tempted to call this an adenocarcinoma. However, thyroid hyperplasia in Lake Erie coho salmon has been reported to be seasonal with a prevalence as great as 93.5% (*10*); lesions even as aggressive as these may still regress if the stimulus or stimuli were removed.



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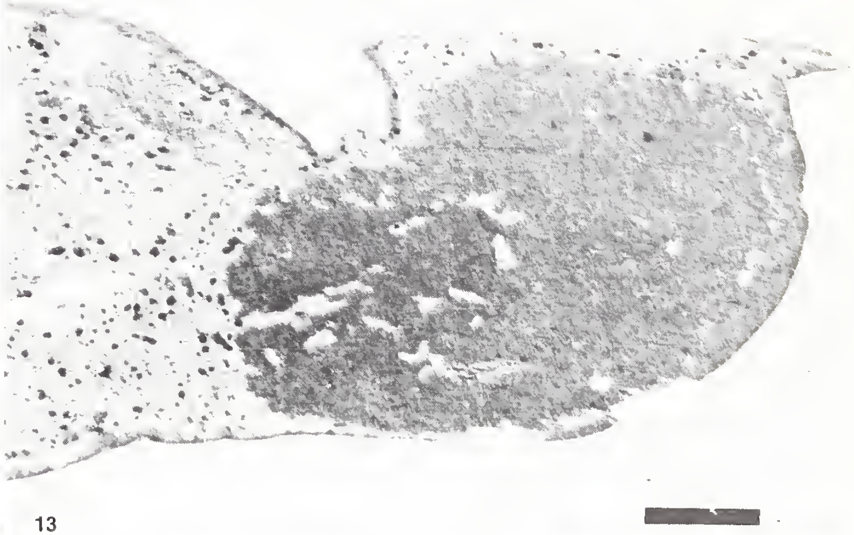

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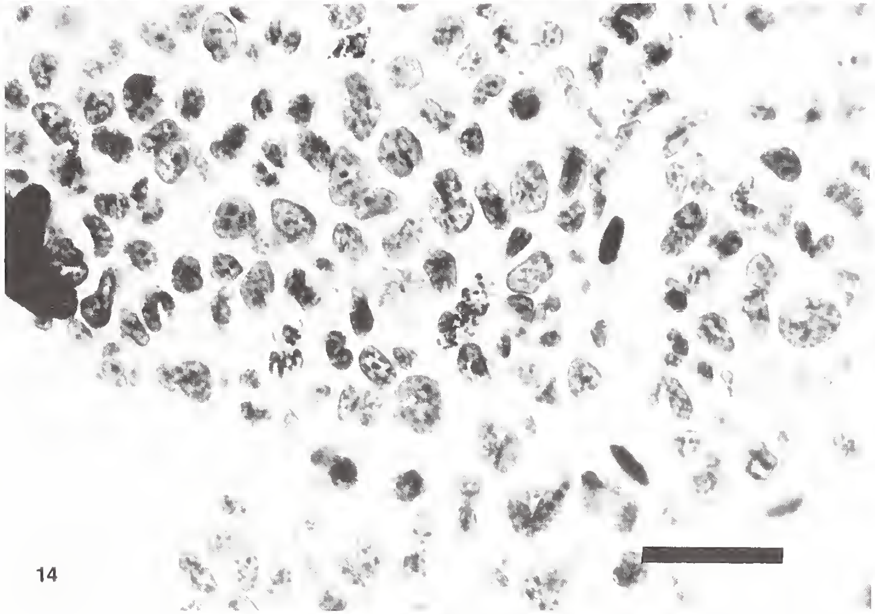

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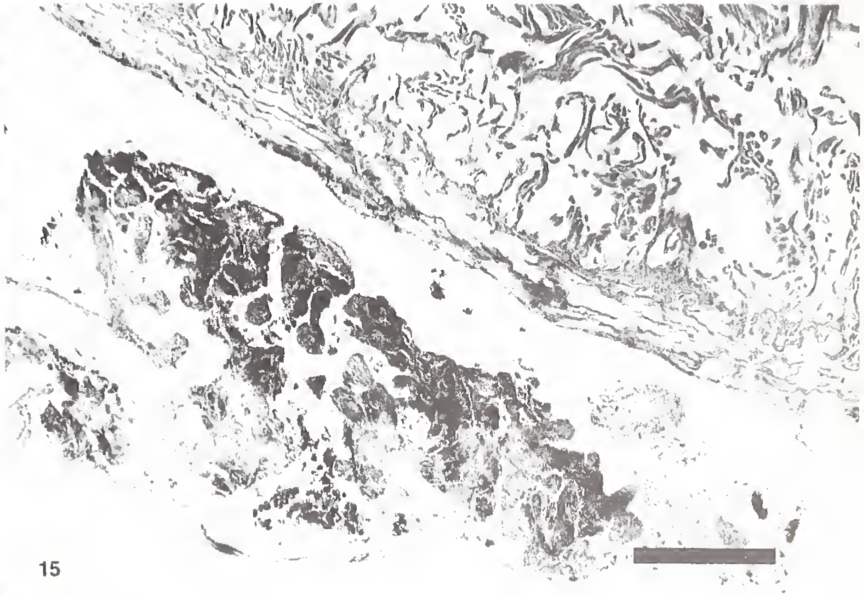

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FIGURE 12.—Focus of normal hematopoiesis (*lower right*) in liver of tiger salamander (RTLA 2468).
FIGURE 13.—Section of figure 12. Dark tissue is normal hematopoiesis. Bar = 1,000 μm.
FIGURE 14.—Detail of figure 13. Mitosis at 8 o'clock position from center. Bar = 30 μm.
FIGURE 15.—Pericardial pouch of normal hematopoietic tissue under heart in Atlantic sturgeon (RTLA 1118). Bar = 500 μm.

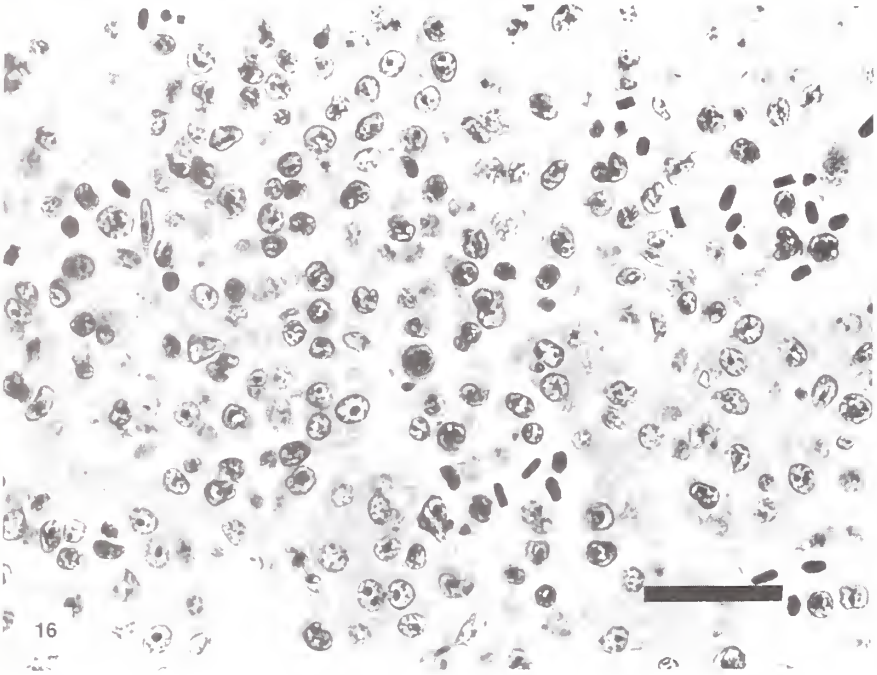

FIGURE 16.—Pernicious anemia vs. granulocytic leukemia in chinook salmon (RTLA 1023). Liver is solid tissue surrounded by erythroblasts or myeloblasts with large, deeply clefted nuclei, prominent nucleoli, and finely granular cytoplasm. Bar = 30 μm.

DYSMORPHOGENESIS VERSUS NEOPLASIA

Dysmorphogenesis results in a spectrum of anomalies that depend on which stage in the development of one individual an attempted duplicative development began for another individual and its relative rate. Siamese twins would result if totipotent cells at the two-cell stage remained partially attached but developed independently at a similar rate. More bizarre monsters or teratoid masses result when pluripotent cells begin development at some later stage. Teratosis is recognized histologically as a collage of normal tissues. Neoplasms arising in pluripotent cells, i.e., teratomas, consist of a collage of abnormal cells of multiple germ layers that are foreign to the site of origin. Hisaoka (*11*) assembled 18 examples of teratoid anomalies in guppies, *Poecilia reticulata*, that represented the full spectrum from Siamese twins to teratomas. Several guppies (RTLA 75; contributed by R. Wenk) had lesions corresponding to some of Hisaoka's. They had a single ventral mass near the pectoral fins and the masses themselves had fins (fig. 23). Histologically, one was comprised predominantly of skeletal muscle (fig. 24) and another, primarily of brain tissue (fig. 25).

Teratoid anomalies contributed by G. W. Piavis and T. Beckerman in sea lampreys, *Petromyzon marinus* (RTLA 2227–2235), arose from the wall of the intestine, the dorsal wall of the coelom, and within the lateral precloacal body wall (*12*).

Among numerous developmental anomalies contributed by J. G. Hnath, one particularly striking example was a portion of body wall from a mature chinook salmon, *Oncorhynchus tshawytscha* (RTLA 1377), with a darkly pigmented area containing an opening (fig. 26). Internally, a gutlike structure originated at the opening and terminated within the mass a short distance away (fig. 27). Histologically, well-differentiated tissues were recognized, including stomach, ceca, nerve, blood-filled heart, and the intestine, which was attached to the anus-like perforation in the body wall.

ADENOFIBROSIS DIFFERENTIATED FROM CHOLANGIOCARCINOMA

Liver lesions, consisting of unencapsulated foci or nodules of multiple ducts and cysts of simple cuboidal or squamous epithelium embedded in a moderate to heavy fibrous connective tissue stroma, have been seen in salamanders and fish from polluted habitats and in fish experimentally exposed to carcinogens. Similar lesions induced in rats were called cholangiofibrosis by Opie (*13*). Stewart and Snell (*14*) called these lesions adenofibrosis because, unlike bile ducts, the glandular lumina contained mucus that reflected a reversion to the ancestral gut epithelium. The presence of metaplastic intestinal cells in rat lesions was verified by electron microscopy (*15*). As adenofibrosis enlarges by peripheral mitoses, liver parenchyma atrophies and recedes (*16*), thereby minimizing the compression of normal liver and significant bulging of the lesions.

In a tiger salamander, *Ambystoma tigrinum* (RTLA 2604; contributed by F. L. Rose), from a sewage settling pond containing perylene, benzpyrene, and other organic hydrocarbons (*17, 18*), enlarging bands of adenofibrosis coalesced and isolated islands of liver in a fairly regular pattern (fig. 28). The lesion consisted of a mat of small tubules enveloped by fibrocytes and collagen (fig. 29). The tubules abutted or were continuous with liver cords without compression or the formation of a fibrous capsule (fig. 30). Because PAS staining was not done, mucus production was unconfirmed.

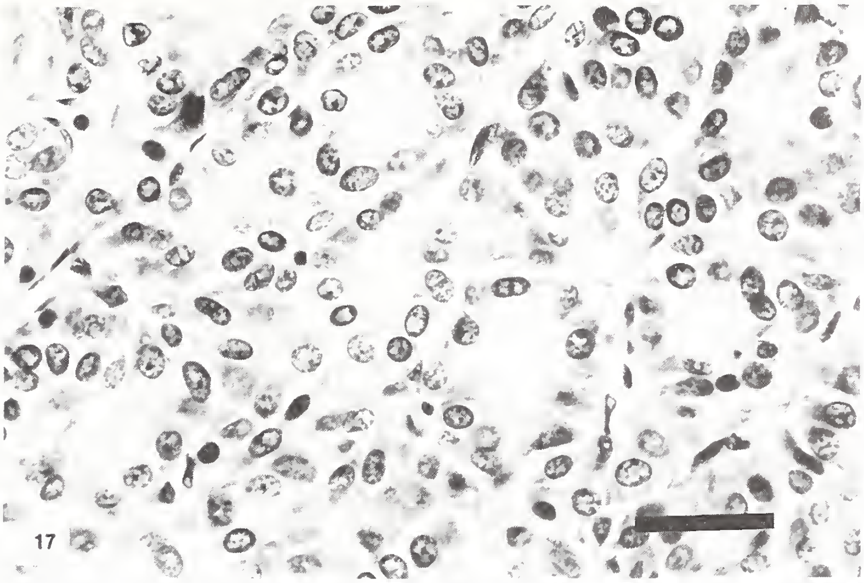

FIGURE 17.—Thyroid hyperplasia/adenocarcinoma in coho salmon (RTLA 2211). Well-differentiated thyroid follicles contain colloid. Bar = 30 μm.

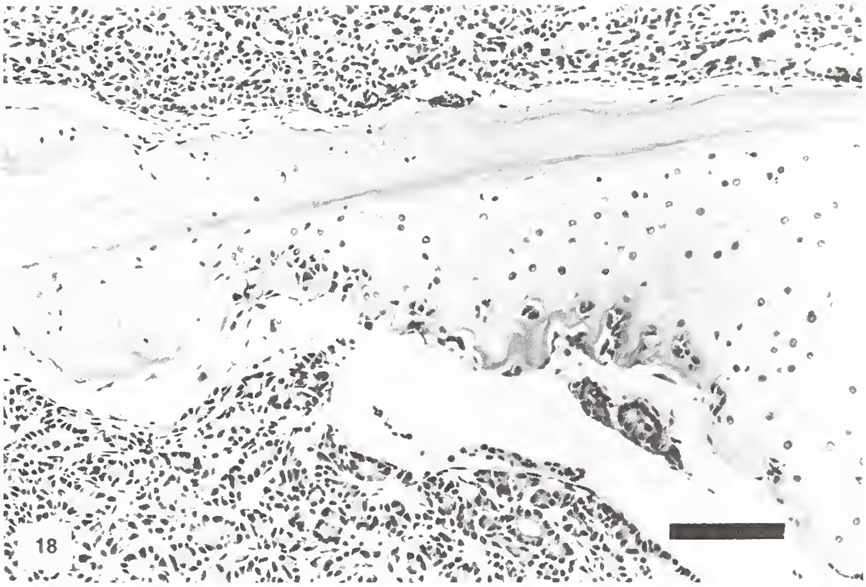

FIGURE 18.—Thyroid (RTLA 2211) etching horizontal band of cartilage. Bar = 100 μm.

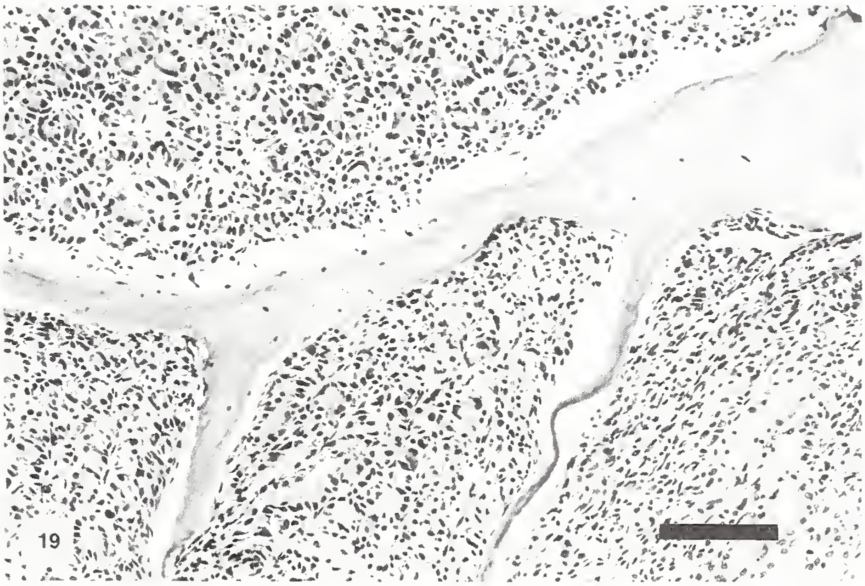

FIGURE 19.—Thyroid (RTLA 2211) etching bone. Bar = 100 μm.

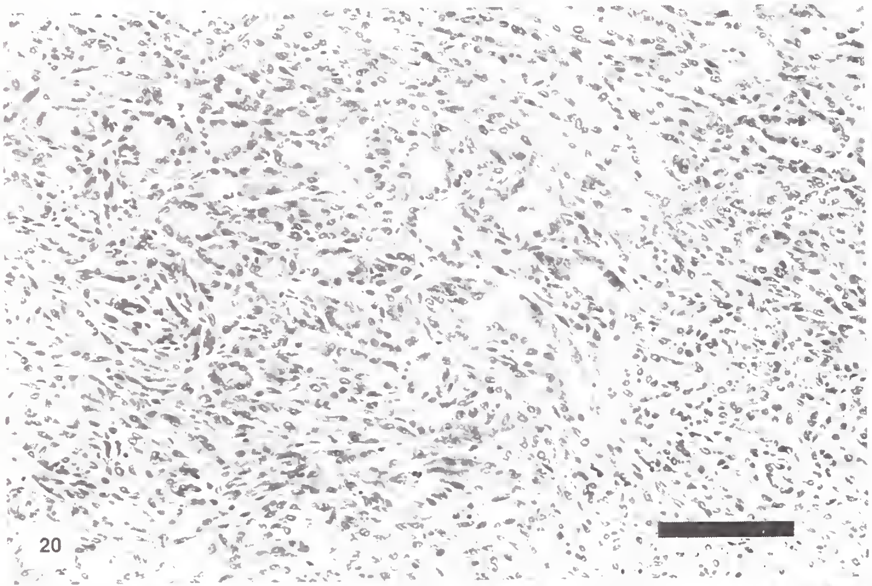

FIGURE 20.—Thyroid (RTLA 2211) follicles not well differentiated. Bar= 30 μm.

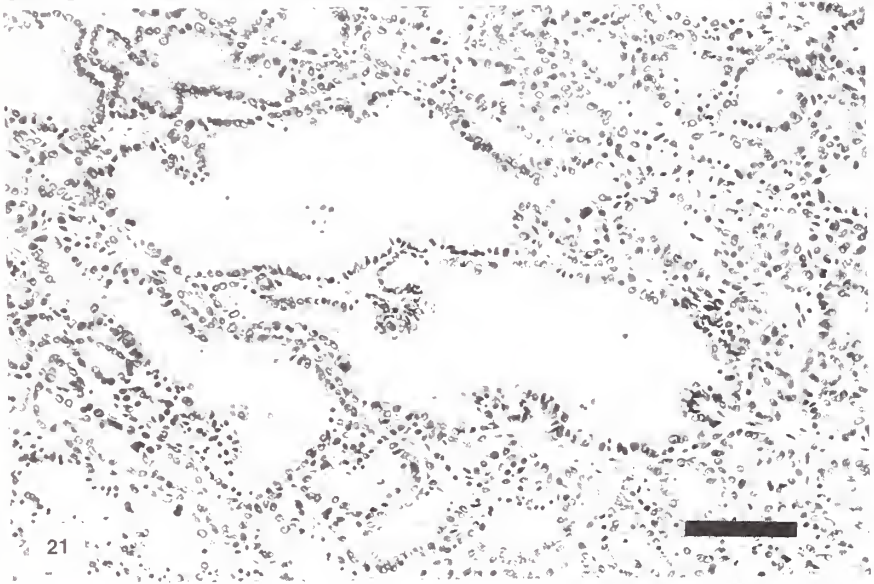

FIGURE 21.—Thyroid (RTLA 2211) with papillary ingrowths into lumina. Bar= 100 μm.

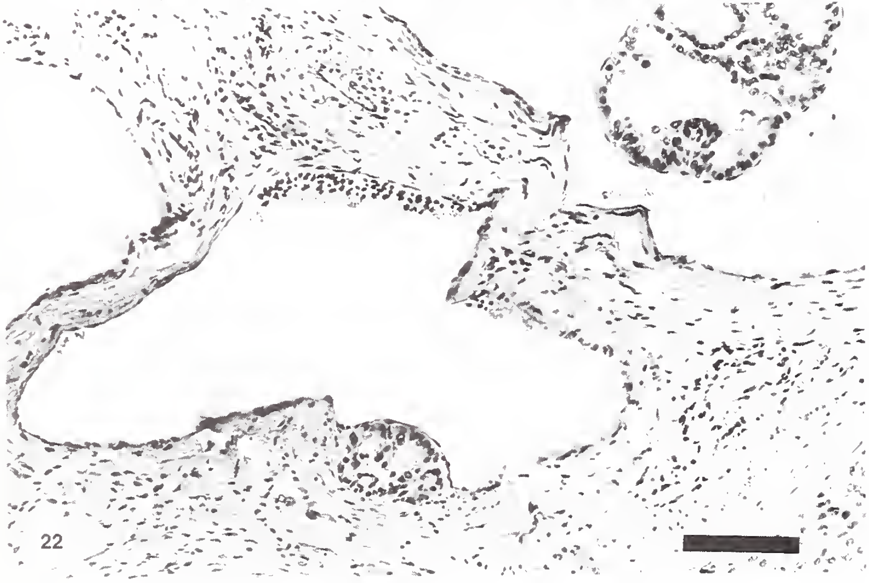

FIGURE 22.—Thyroid (RTLA 2211) with emboli in blood vessel (*top right*) and growing from vessel wall (*bottom center*). Red blood cells inside central vessel along wall *at top*. Bar= 100 μm.

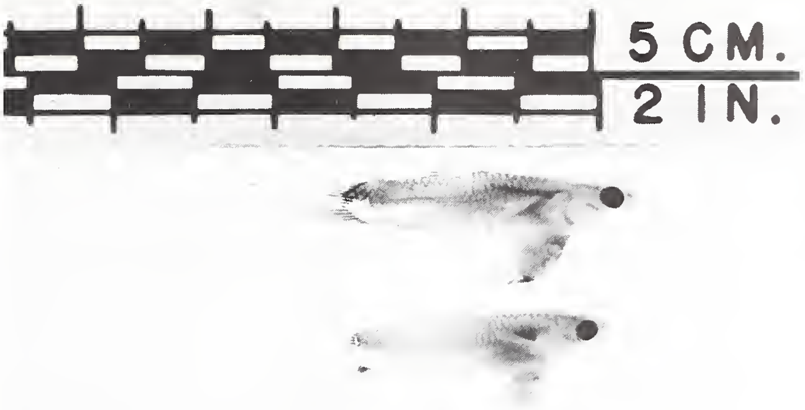

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FIGURE 23.—Teratoid anomalies in guppies (RTLA 75). Structure streaming caudally from tip of lesion of top fish is a fin.

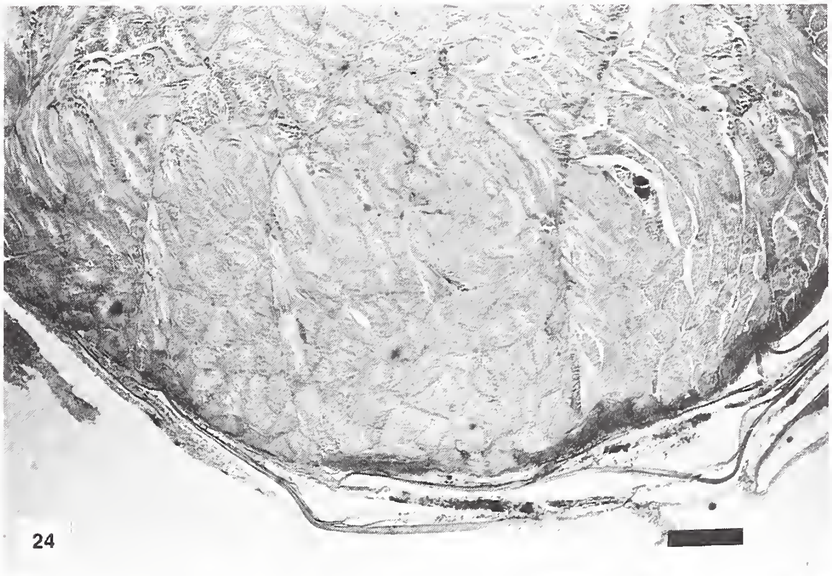

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FIGURE 24.—Teratoid anomaly (RTLA 75). Ventral protrusion is skeletal muscle covered by skin. Bar = 500 μm.

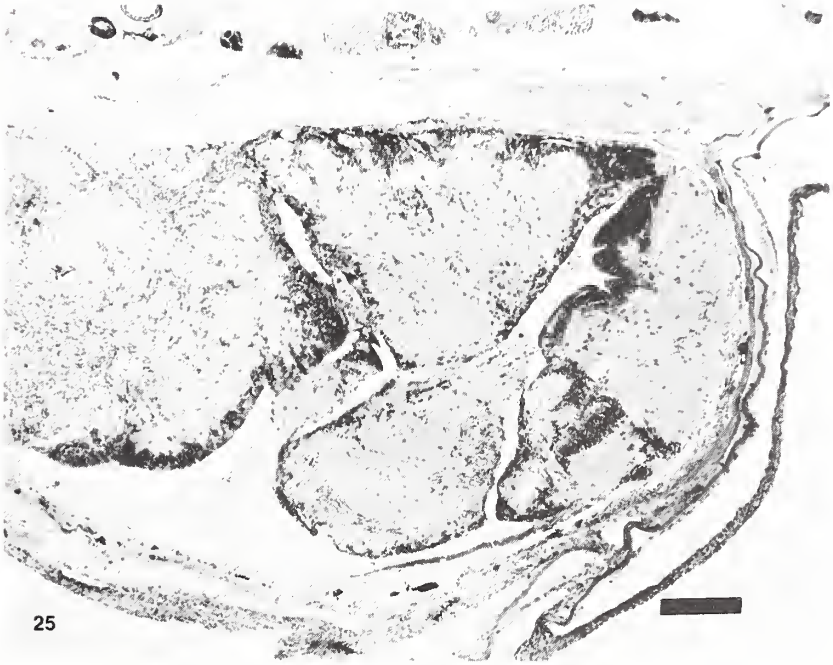

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FIGURE 25.—Teratoid anomaly (RTLA 75). Ventral protrusion is brain tissue covered by skin. Nonteratogenic testis above lesion. Bar = 500 μm.

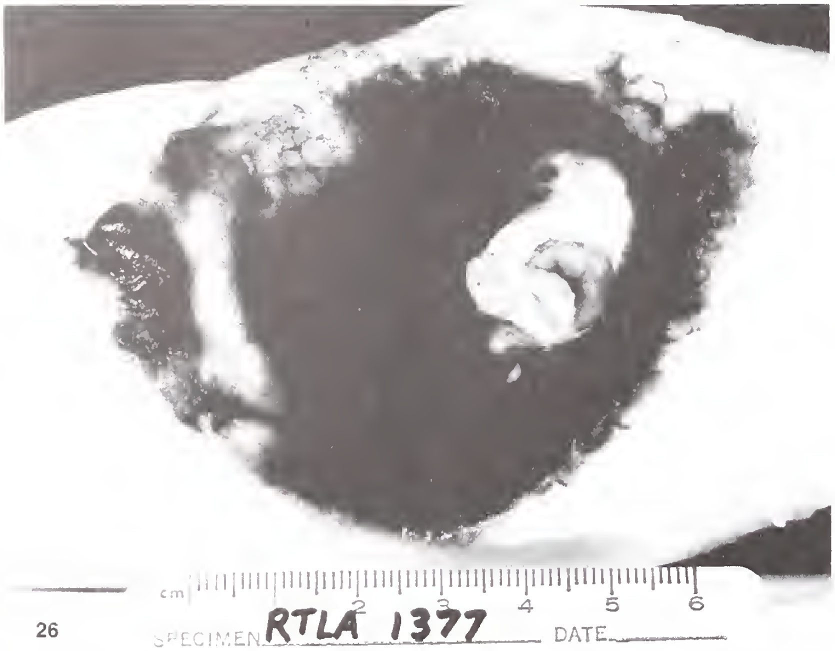

FIGURE 26.—Teratoid anomaly in chinook salmon (RTLA 1377). Anus-like opening in pigmented area on outside body wall.

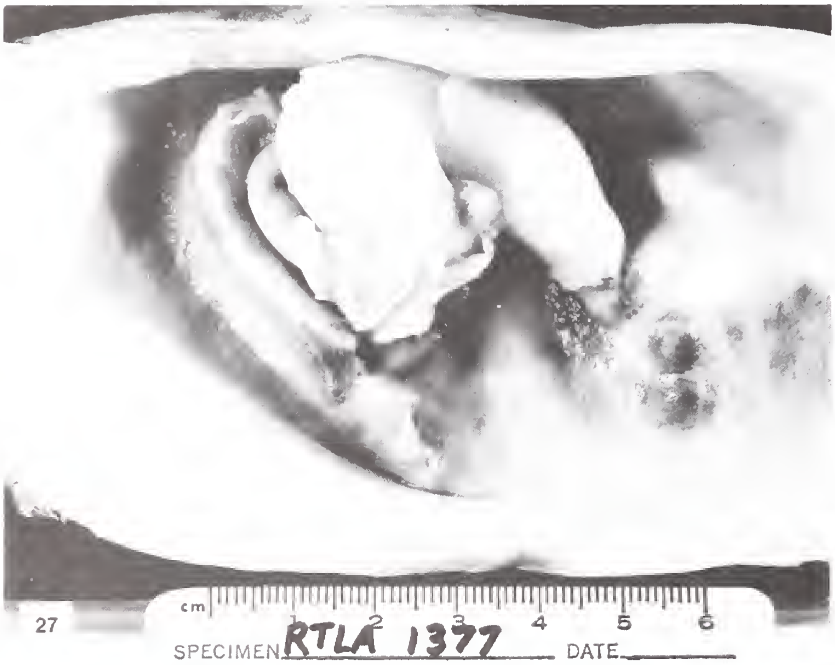

FIGURE 27.—Gutlike structure attached to inside of body wall shown in figure 26 (RTLA 1377).

In adenofibrosis seen in several fish species, tubular elements were variably larger than illustrated for the tiger salamander and in some species formed small cysts. The fibrous component varied from a few layers of cells outlining tubules and cysts to a thick peritubular matrix comprising the bulk of the lesion. Cholangiomas in white suckers, *Catostomus commersoni* [RTLA 7; contributed by C. J. Dawe, M. F. Stanton, F. J. Schwartz; (*19*)], were well-circumscribed but unencapsulated, with well-differentiated tubules and moderate fibrous stroma. Tubules did not undergo transition into liver cords. Cholangiocarcinomas in brown bullheads, *Ictalurus nebulosus* (RTLA 2512, 2513; contributed by P. C. Baumann), were moderately to poorly differentiated, had variable amounts of stroma, and invaded liver parenchyma. Mitotic activity was more pronounced at the periphery of larger lesions. Small lesions had well-differentiated tubular elements resembling adenofibrosis. Based on these observations, adenofibrosis in the livers of amphibians and fish consists of bile ductlike tubules, possibly metaplastic, in a concentric fibrous matrix. Cholangiocarcinomas in fish are poorly differentiated and invasive. Intermediate stages suggest that some

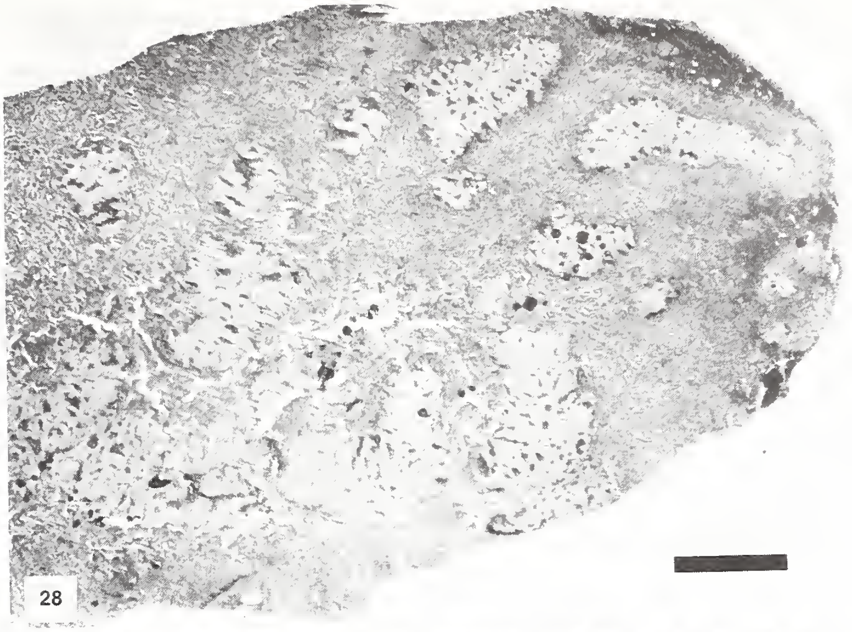

FIGURE 28.—Adenofibrosis in liver of tiger salamander (RTLA 2604). Islands of liver (light tissue) isolated by bands of adenofibrosis (dark tissue). Bar = 1,000 μm.

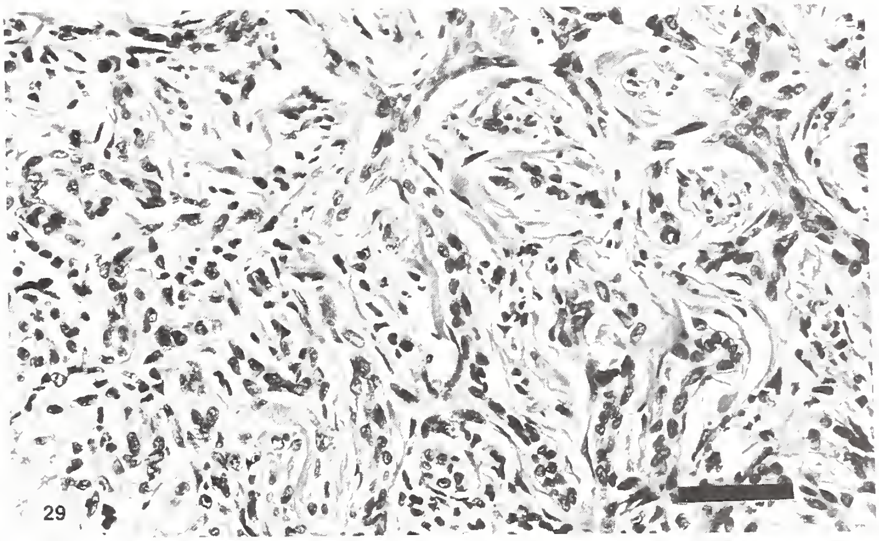

FIGURE 29.—Adenofibrosis (RTLA 2604). Matrix of bile ductules in a fibrocyte and collagen stroma. Bar = 100 μm.

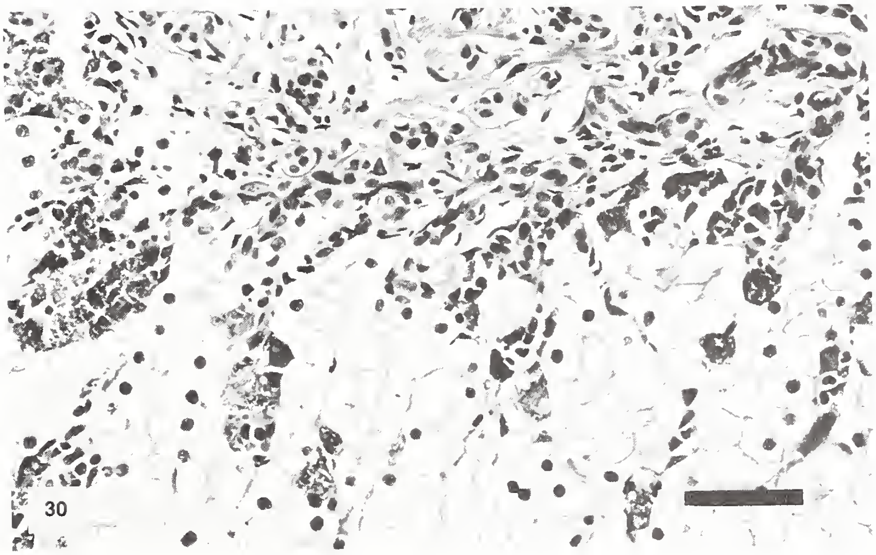

FIGURE 30.—Adenofibrosis (RTLA 2604) transitions into normal liver (*bottom*) without compression or encapsulation. Bar = 100 μm.

lesions may start as adenofibrosis and progress into neoplasia. This possibility is controversial even in rodents (*16, 20, 21*) and needs experimental verification.

LYMPHOCYSTIS

Lymphocystis lesions of bony fish, although long known to be non-neoplastic, have been called "tumors" over much of the 108-year history of the disease (*22, 23*) because of their tumor-like gross appearance. A case of lymphocystis in a starry flounder, *Platichthys stellatus* (RTLA 504; contributed by B. F. Deys), serves as an example. Gross lesions were multiple, round, birdshot to pea-sized, unpigmented growths predominantly seen in the webbing of fins but also on any area of the skin (figs. 31, 32). Microscopically, individual lesions consisted of a pouch of normal epidermis distended above the dermis, containing the enormous lymphocystic cells, some exceeding 0.5 mm in diameter (fig. 33); cells of 1.5 mm have been reported (*24*). These enormous cells, once thought to be insect ova or protozoa, are hypertrophied fibroblasts in response to submicroscopic infectious agents (*25*); subsequent morphological characterization (*26*) and isolation (*27*) indicated that the agent was cytoplasmic DNA iridovirus. Mature lymphocystis cells (fig. 34) are surrounded by a hyalin capsule and contain a fine granular cytoplasm exhibiting basophilic inclusions, a large nucleus with irregular nuclear membrane, and a proportionally enlarged nucleolus (not shown). A reactive, thin fibrous capsule often forms around the lymphocystic cells, and the size differential of the 2 cell types demonstrates the incredible degree of hypertrophy.

GLOCHIDIOSIS

Few neoplasms of gill epithelium are known in fish or amphibians, and only one in mollusks has been identified (*28, 29*); this is a local epizootic gill carcinoma in the clam, *Macoma balthica* (RTLA 1004). However, focal and diffuse idiopathic gill epithelial hyperplasia is fairly often found in both fish and bivalve mollusks collected in natural

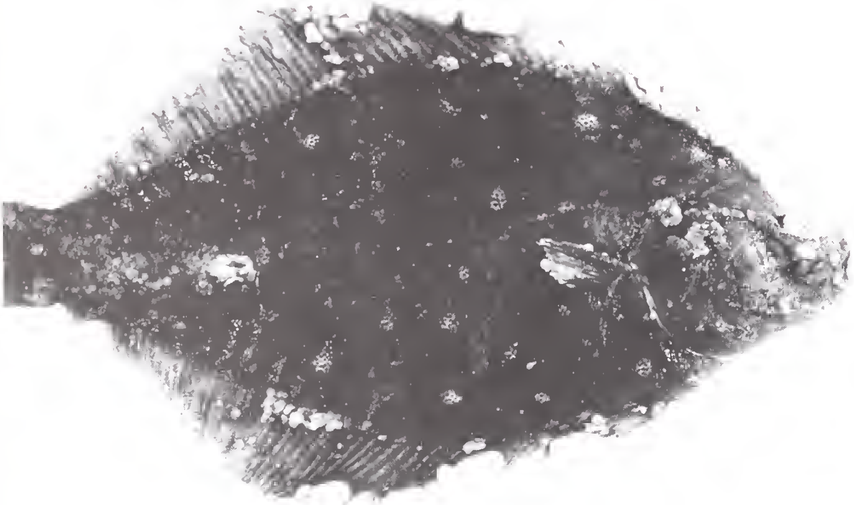

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FIGURE 31.—Lymphocystis in starry flounder (RTLA 504). Lesions in webbing of fins and on skin of eyed-side.

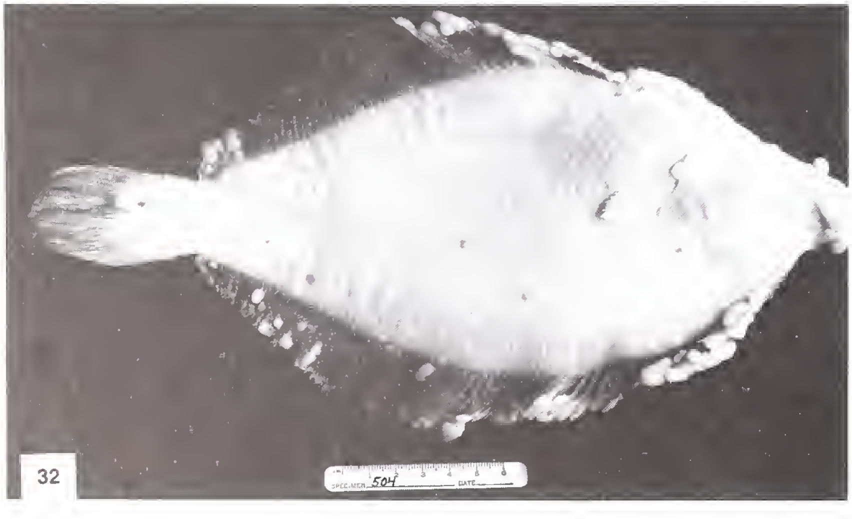


FIGURE 32.—Lymphocystis (RTLA 504). Lesions in webbing of fins and on skin of blind side.

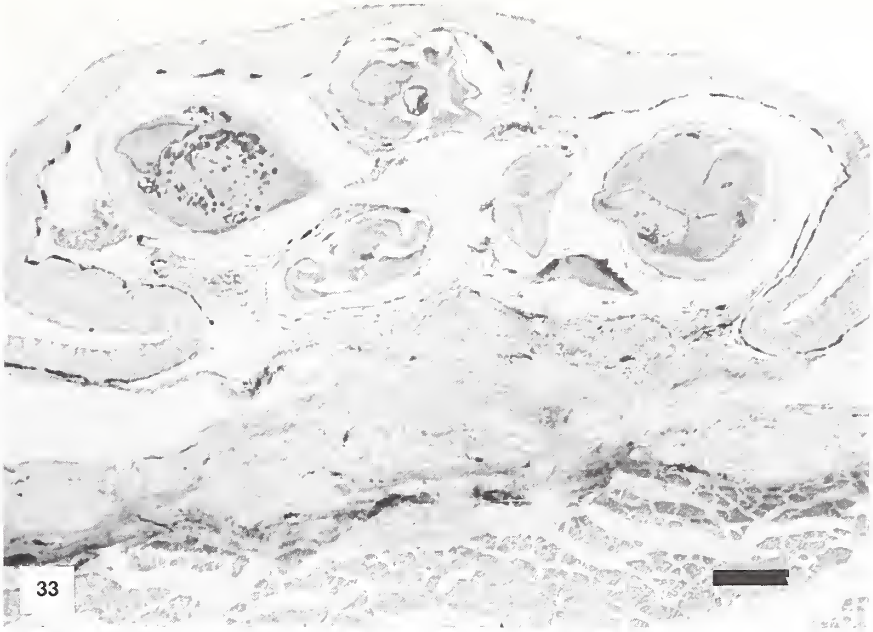


FIGURE 33.—Lymphocystis (RTLA 504). Subepidermal pouch containing lymphocystis cells. Bar = 500 μm.

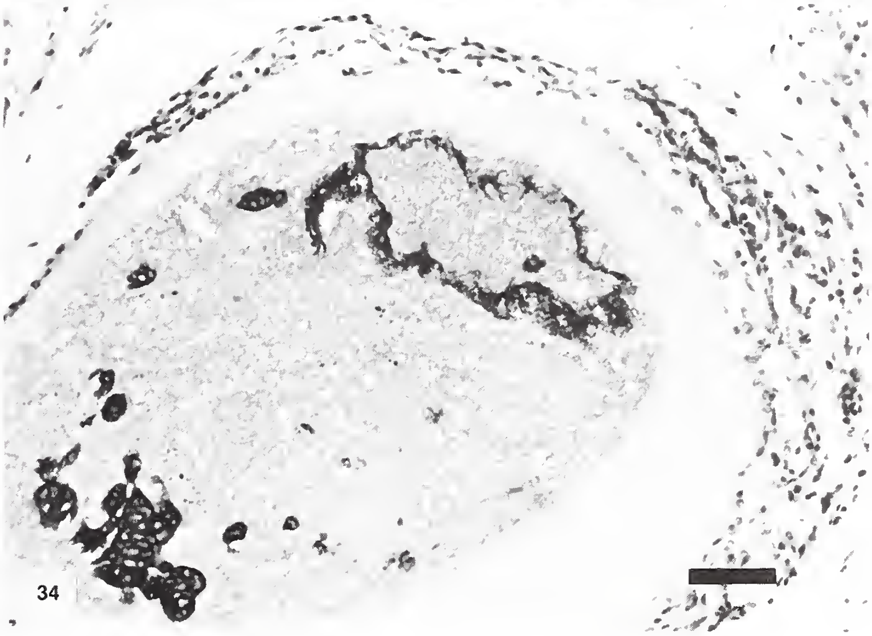


FIGURE 34.—Lymphocystis (RTLA 504). Mature lymphocystis cell with hyalin capsule, granular cytoplasm with basophilic inclusions, large nucleus with irregular membrane (*upper right*) and reactive thin fibrous capsule of normal-sized cells. Bar = 30 μm.

habitats. Experimentally, fish gill epithelial hyperplasia has been induced by various chemicals (*30*) and by glochidia (*31*), larvae of freshwater mussels that spend a portion of their life cycle ranging from several weeks to 1 year encysted in gills and sometimes fins of fish.

The gills of coho salmon (*Oncorhynchus kisutch*) experimentally infected with glochidia of *Margaritifera margaritifera* (RTLA 1119; contributed by T. R. Meyers) had multiple focal lesions consisting of several layers of gill epithelium surrounding each mussel larva (fig. 35). In the report of this study (*31*), some fish were more permissive hosts than others. Permissive hosts completed encystment by hyperplastic migrating gill epithelium, without other tissues being involved, in 8-16 hours. In less permissive fish, most larvae had been sloughed by 16 hours. The few cysts that formed became the target of continued epithelial hyperplasia forcing glochidial cysts from affected gill filaments. Both types of fish hosts also contained residual parasite-free hyperplastic nodules comprised of epithelial cells and fibroblastic elements.

REACTIVE FIBROSIS VERSUS FIBROMA

Some hosts produce reactive fibrous tissue continuously in response to viable parasites that ultimately results in enormous fibrous masses. Even multiple tissue blocks from such a mass may miss the parasites, their ova, or other products; if discovered, the ratio of parasite-to-host tissue

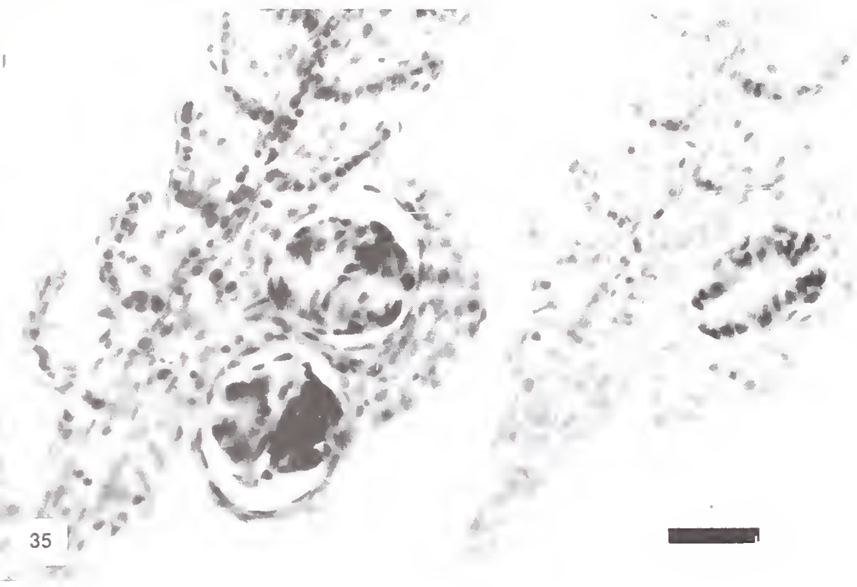


FIGURE 35.—Glochidiosis in gill of coho salmon (RTLA 1119). Epithelium of 2 gill filaments has encysted 3 glochidia (larvae) of a freshwater mussel. Bar = 30 μm.

may be so small as to appear insignificant or adventitious, which could lead to an erroneous diagnosis of well-differentiated fibroma or one of its variations.

Such lesions may be represented by the wartlike growths approaching 1 kg in weight (figs. 36, 37) and occurring on all exposed surfaces from the tail to the eyelids of green sea turtles, *Chelonia mydas* (RTLA 12, 121, 1883; contributed by G. H. Waddell, A. C. Smith, and G. H. Balazs, respectively). Small growths were rounded with a setose surface. Observed histologically, they consisted of moderately cellular, moderately well-vascularized fibrous tissue covered by hyperplastic keratinized epidermis (fig. 38). Large growths were rugose with a hyperplastic keratinizing epidermis similar to the small lesions but with fewer pegs and papillae. Underneath the epidermis, the large lesions consisted of sparsely cellular, collagen-rich, monotonous fields of wavy fibrous tissue (fig. 39). Shortly after their initial description (*32*), trematode ova were observed in the vascular bed of the growths (*33*), and leeches were noted on their surfaces (*34*).

The disease was recently reported to behave as if infectious in mariculture populations of *C. mydas* in the West Indies (*35*). Jacobson's evaluation by electron microscopy failed to demonstrate a viral etiology, nor was Sing Chen Chang of the RTLA successful in his attempts to do so. However, the heart and great vessels of the Cayman Island turtles were infested with the spirorchid trematode, *Laeredius learedi,* whose ova were found in various tissues including dermal capillaries. Investigators have speculated about the effects of the anticoagulant hirudin injected into the growths by ectoparasitic leeches, *Ozobranchus* sp., but no effects were established. Although several species of snails were found in the culture tanks, they could not be verified as alternate hosts for the trematodes.

With the infectious behavior, apparent absence of viruses, presence of trematode ova, and defensive nature of fibrous tissue, the consensus is that these lesions represent reactive fibrosis instead of fibromas and fibropapillomas as previously thought.

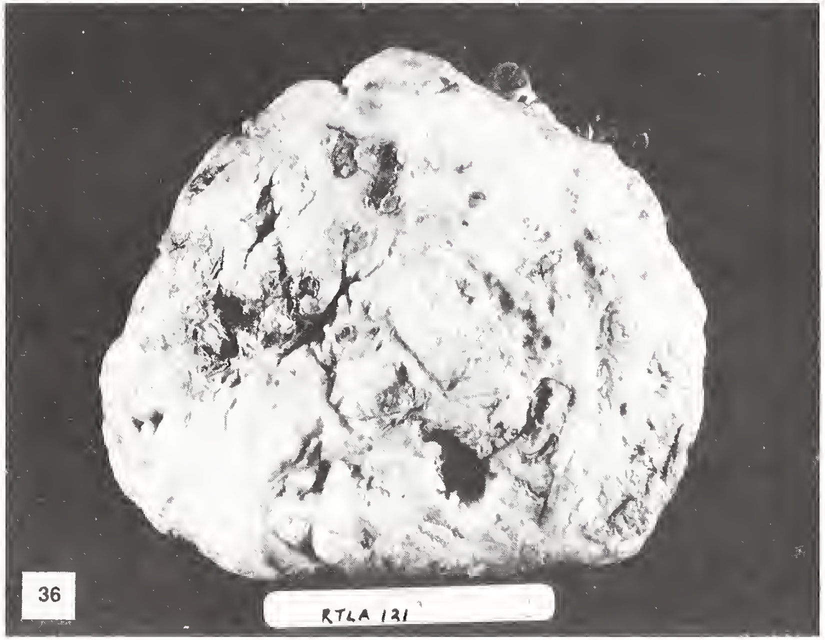
BLACK SPOT DISEASE DIFFERENTIATED FROM MELANOPHOROMA

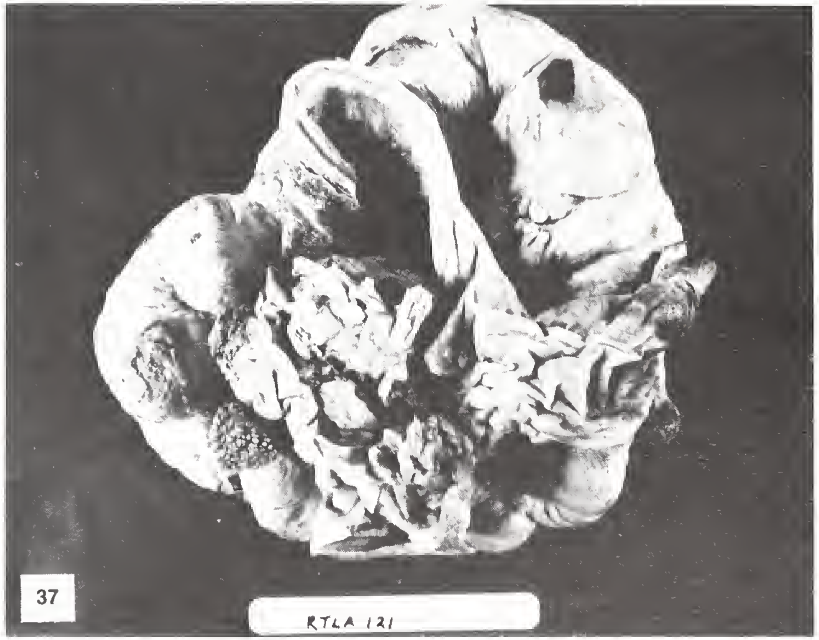
A live northern pike, *Esox lucius* (RTLA 937; contributed by E. R. Brown), had 1- to 4-mm pigmented protrusions scattered over the entire body and inside the mouth. Microscopically, trematode metacercariae in the dermis were encapsulated first by a band of inflammatory leukocytes followed by fibrous tissue containing melanophores (fig. 40), a well-known condition called "black spot," which is caused by digenean metacercariae from several genera (*23*).

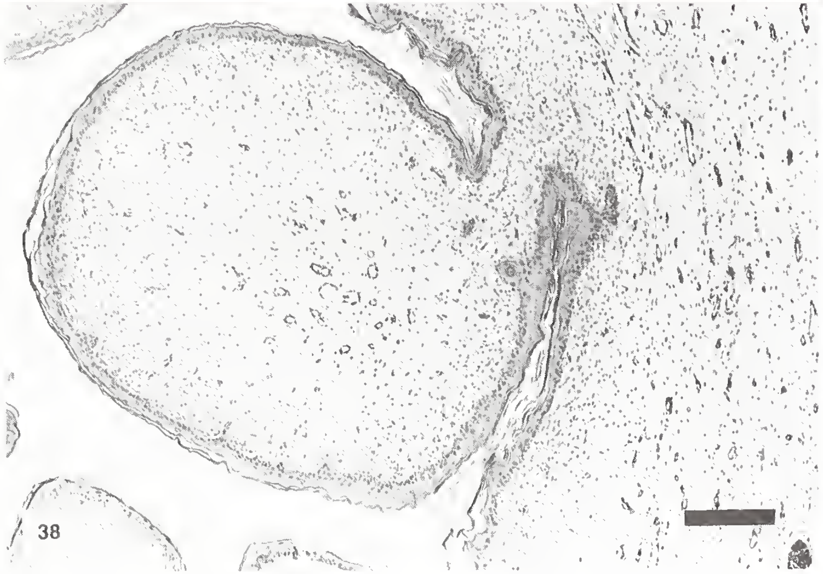
Another type of pseudomelanophoroma has been seen in bullheads. Small, black lesions on the skin resembling nevi resulted from a concentration of normal melanophores, as epidermal cells piled up during papillary epidermal hyperplasia or neoplasia.

PARASITIC LEUKOCYTOSIS VERSUS PARASITIC MONOCYTIC LEUKEMIA

A disease outbreak in cultured turbot, *Scophthalmus maximus* (RTLA 842; contributed by R. J. Roberts), was characterized grossly by subcutaneous and visceral masses up to 4 cm. Microscopically, masses appeared to be homogeneous populations of cells containing random, small foci of necrosis. Cells lined up along vessels and fibers, and adjacent normal tissue was infiltrated (fig. 41). The cells were monocytic with lobulated nuclei and fairly abundant cytoplasm. At high magnification, most cells contained one to several, PAS-positive, oval to lancet-shaped parasites (fig. 41, inset), which were identified by electron microscopy (*36–38*) as trophozoites of *Haemogregarina sachai.* The monocytic cells were macrophages at several levels of maturation with parasitized neutrophils







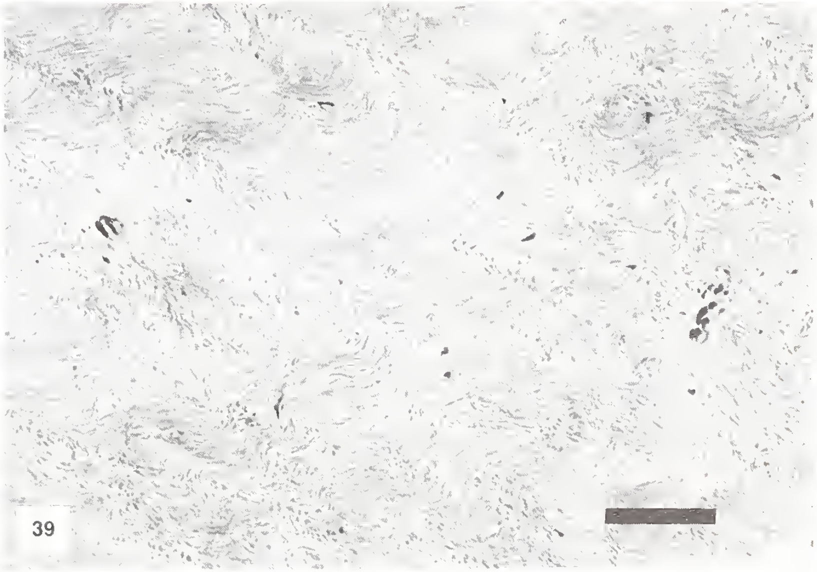


FIGURE 36.—Fibrosis vs. fibropapilloma in green sea turtle (RTLA 121). Dorsal side of 750-g lesion.
FIGURE 37.—Underneath view of figure 36. Early setose lesions (margin at 4 and at 7 o'clock positions).
FIGURE 38.—Fibrosis in green sea turtle (RTLA 12). Early lesion with moderately cellular, moderately well-vascularized fibrous tissue bordered by keratinizing epithelium. Bar=200 μm.
FIGURE 39.—Fibrosis (RTLA 12). Old lesion with sparsely cellular, collagen-rich, monotonous field of fibrous tissue. Bar=100 μm.

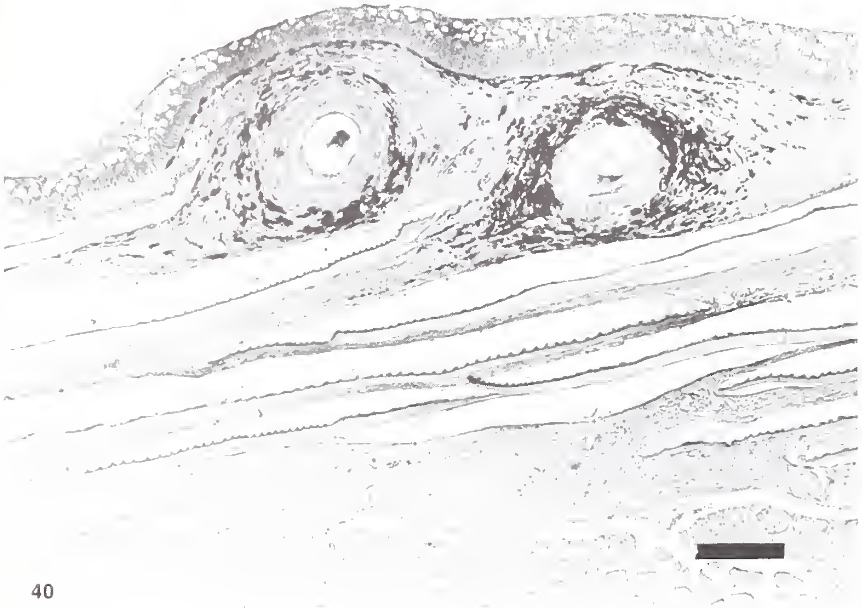


Figure 40.—Black spot disease in northern pike (RTLA 937). Two parasitic granulomas (*center left* and *right*). Core of left granuloma has vestige of trematode parasite. Concentric tissue adjacent to core consists of epithelioid monocytes. Pigmented melanophores are concentrated in dermal fibrous capsule. Normal epidermis (*top*). Barbed scales *underneath*. Bar = 100 μm.

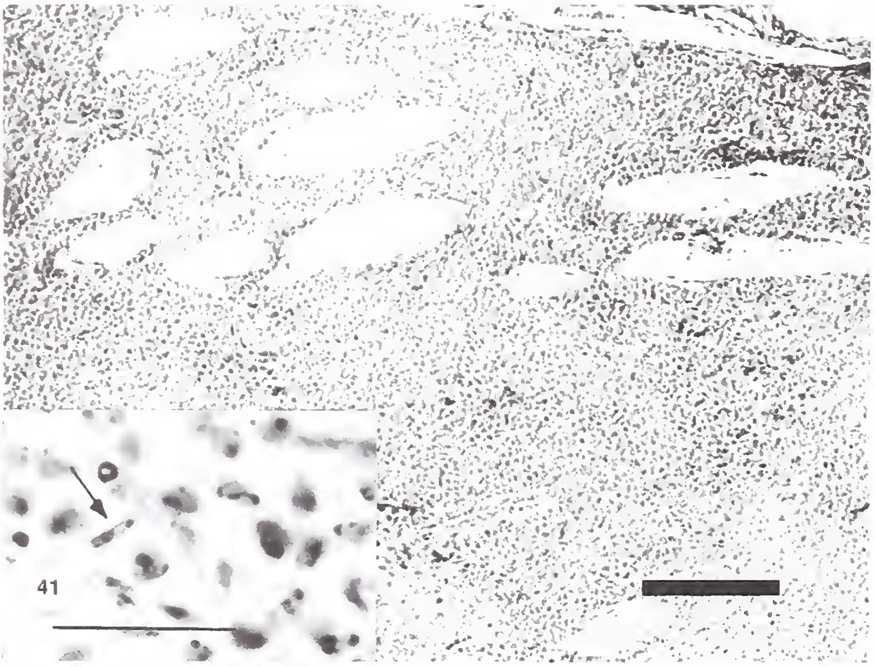


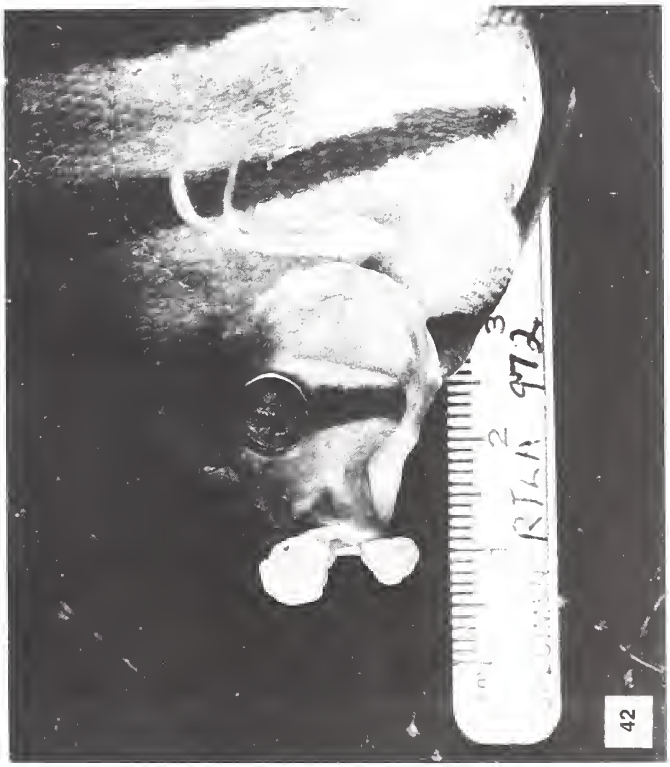
Figure 41.—Parasitic leukocytosis in turbot (RTLA 842). Field of monocytic cells enveloping muscle bundles. Bar = 100 μm. Inset: Virtually every cell contains from one to several ciliates. *Note* lancet shape of parasite (*arrow*). Bar = 15 μm.

Figure 42.—Reactive hyperplasia with tooth formation vs. odontoma in angelfish (RTLA 972). Lesions on both lips.

Figure 43.—Reactive hyperplasia vs. odontoma in angelfish (RTLA 2189.1). Lesions are characteristically symmetrical and consist of loose connective tissue. Dark radiating spicules are bone. Surface epithelium is clefted with enamel organs (barely visible) forming at base of some clefts. Bar = 1,000 μm.

Figure 44.—Reactive hyperplasia with tooth formation vs. odontoma (RTLA 972). Deep epithelial clefts, some forming enamel organs (*top center* and *top left*). Loose connective tissue contains bone spicules (*left* of *center*). Bar = 500 μm.

Figure 45.—Reactive hyperplasia vs. odontoma (RTLA 2189.2). Inflammatory leukocytes disseminated within loose connective tissue. Bar = 100 μm.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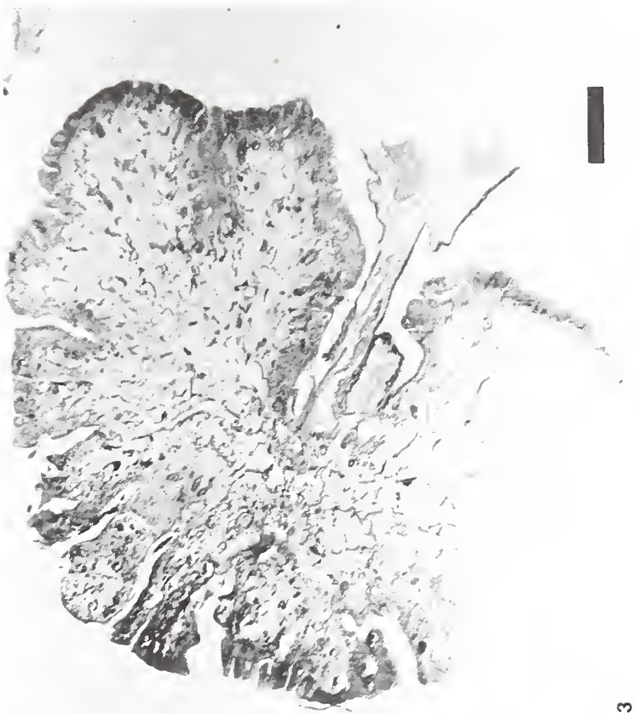

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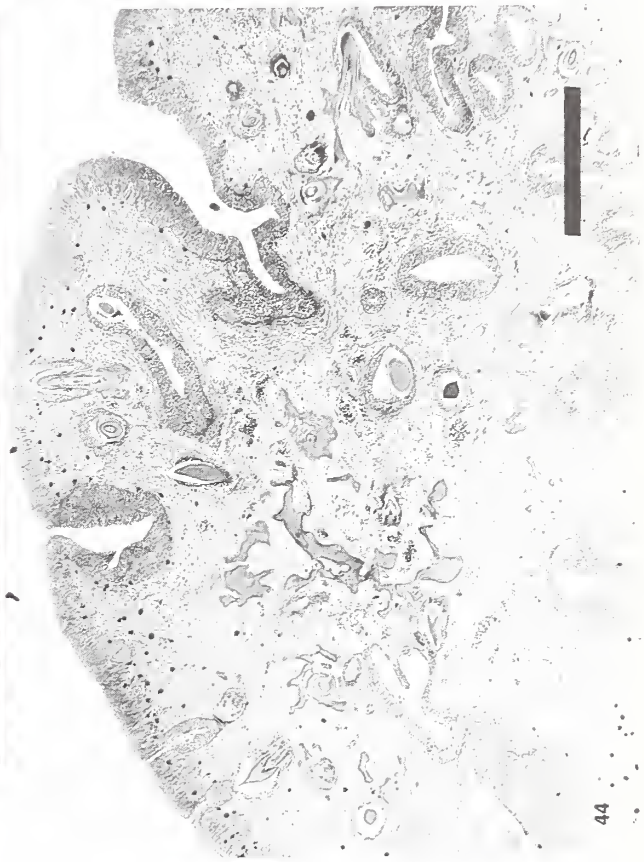

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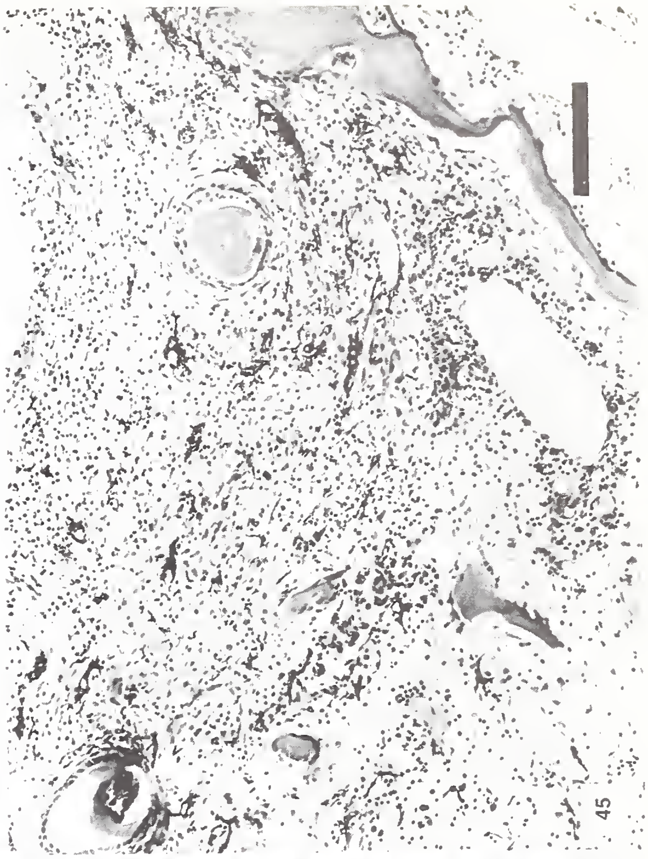

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and lymphocytes also present. Initially, the diagnosis of parasitic leukosis was considered, but this was ruled out later by further studies in favor of a parasitic leukocytosis.

REACTIVE HYPERPLASIA WITH TOOTH FORMATION DIFFERENTIATED FROM ODONTOMA

Angelfish, *Pterophyllum scalare* (RTLA 972, 2189, and 2263; contributed by P. M. Hine, G. C. Blasiola, Jr., and G. Migaki, respectively), had bulbous, granular lesions protruding from one or both lips (fig. 42). Tissues radiated symmetrically from a narrow point of attachment (fig. 43). Lesions consisted of loose, spongy, fibrous connective tissue containing fragments of new bone radiating from the mandible and maxilla at the point of attachment. Peripherally, they contained numerous teeth in all stages of well-formed development properly oriented toward the surface (fig. 44). Inflammatory leukocytes were present in varying numbers throughout (fig. 45). The surfaces of lesions were covered by a layer of epithelium which had numerous clefts where pegs extended into underlying connective tissue. Some pegs were modified into enamel organs consistent with normal tooth formation (fig. 46). In the normal formation of fish teeth, as in mammals, pegs of oral epithelium grow into the connective tissue, enlarge and evert at the apex forming a 2-layer, bell-shaped enamel organ. Ameloblasts at the opening of the enamel organ induced connective tissue inside the bell to differentiate into odontogenic pulp bounded by a layer of dentin-secreting odontoblasts. Ameloblasts on the inside surface of the bell secrete an enamel crown to overlay the dentin. The crown then erupts through the skin surface. Unlike mammals, fish continually shed and replace their teeth.

In summary, the lesions were roughly symmetrical and consisted of a loose, probably edematous, fibrous stroma (which contained metaplastic spicules of bone, inflammatory leukocytes, and peripheral well-formed teeth) and were covered by epithelium. No unequivocally neoplastic tissue was readily apparent, in contrast to odontogenic neoplasms reported in other fish species (*39, 40*) which have been highly cellular, embryonal growths ranging, in architecture, from anaplastic to organoid. Furthermore, these angelfish specimens were held captive in glass aquaria. Puffy snouts, presumably from banging into the glass walls, are fairly common in certain captive fish as are readily seen at public aquaria. Despite the tumor-like gross appearance and the production of large numbers of teeth, this combination of features is more suggestive of a reactive hyperplasia with tooth formation due to trauma than of an odontogenic neoplasm.

CHRONIC INFLAMMATION

A dwarf-clawed frog, *Hymenochirus boettgeri* (RTLA 420; contributed by R. Wenk), had a soft, amorphous lesion under the mandible. Microscopically, the lesion was highly cellular (fig. 47), with most cells containing a foamy cytoplasm and nuclear features that suggested they were macrophages. However, small collections of lymphoid cells were also seen. A Ziehl-Neelsen-stained slide revealed large numbers of acid-fast bacteria (fig. 48). Acid-fast bacteria are ubiquitous around amphibians (*41*) and result in many inflammatory lesions such as this one, which may be mistaken for lymphoid and other types of neoplasms (*42*).

GRANULOMATOUS INFLAMMATION

Metazoan animals at all phylogenetic levels produce focal, chronic inflammatory reactions with variable complements of cell types, necrosis, and stromal proliferation to injured tissue regardless of cause, e.g., microbial, toxic, metabolic, or traumatic, and to immunologically reactive exogenous substances. Phagocytes predominate in the initial reaction, although, in vertebrates, granulocytes and

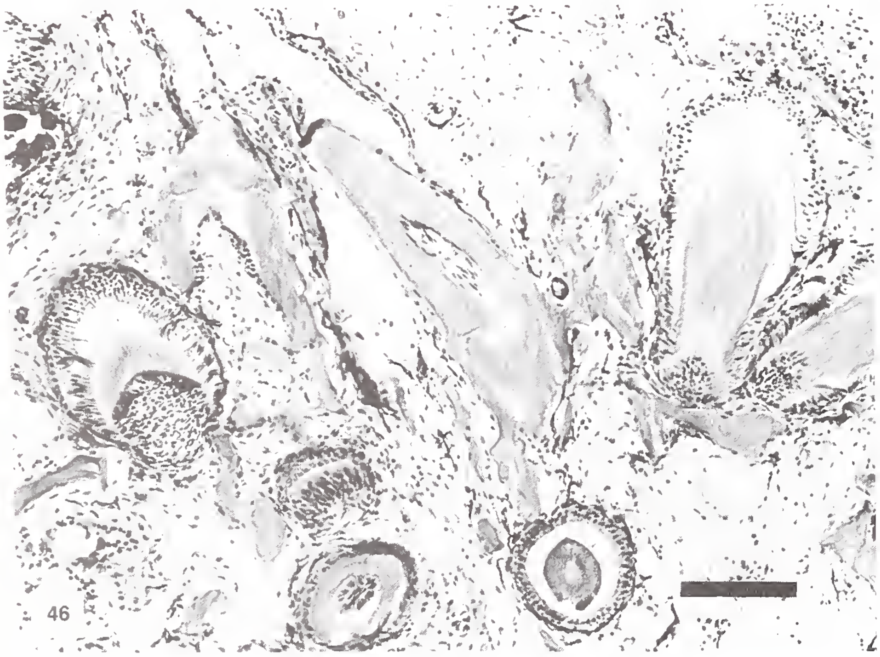

FIGURE 46.— Reactive hyperplasia vs. odontoma (RTLA 2189.2). Teeth in several stages of development. Developing tooth (*left center*) has outer double-layered, bell-shaped, enamel organ; the inner layer of ameloblasts presumably had produced enamel which dissolved during processing and left clear space. Crescent-shaped acellular material under the clear space is dentin, produced by odontoblasts lining the pulp of the dental papilla. Cross section of tooth (*bottom right*) has outer 2-layered enamel organ enclosing on empty enamel space surrounding a central core of dentin. Deeper cross section (*bottom left*) went through tip of odontoblast-lined papilla (*center*). Bar= 100 μm.

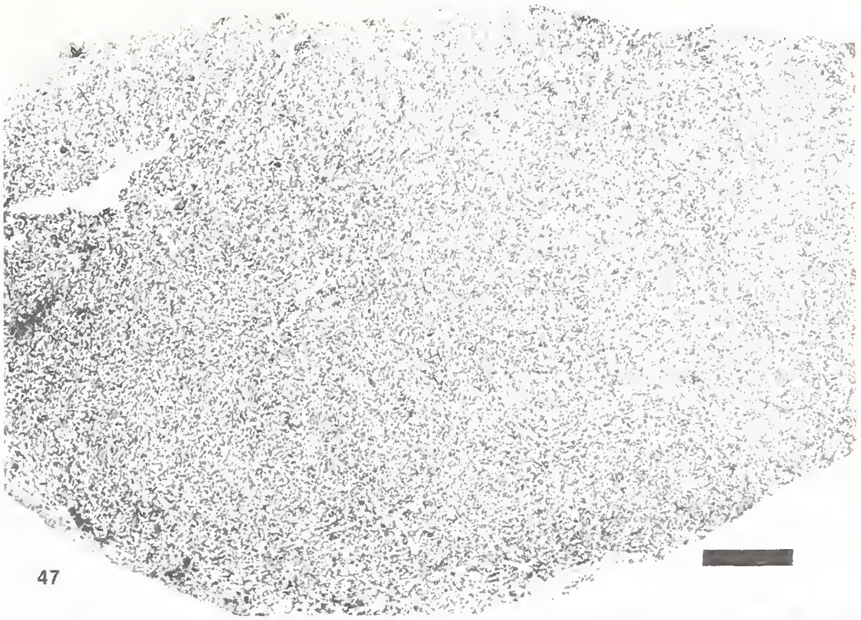

FIGURE 47.—Chronic inflammation to acid-fast bacteria in a dwarf-clawed frog (RTLA 420). Highly cellular mass under jaw. Bar=200 μm.

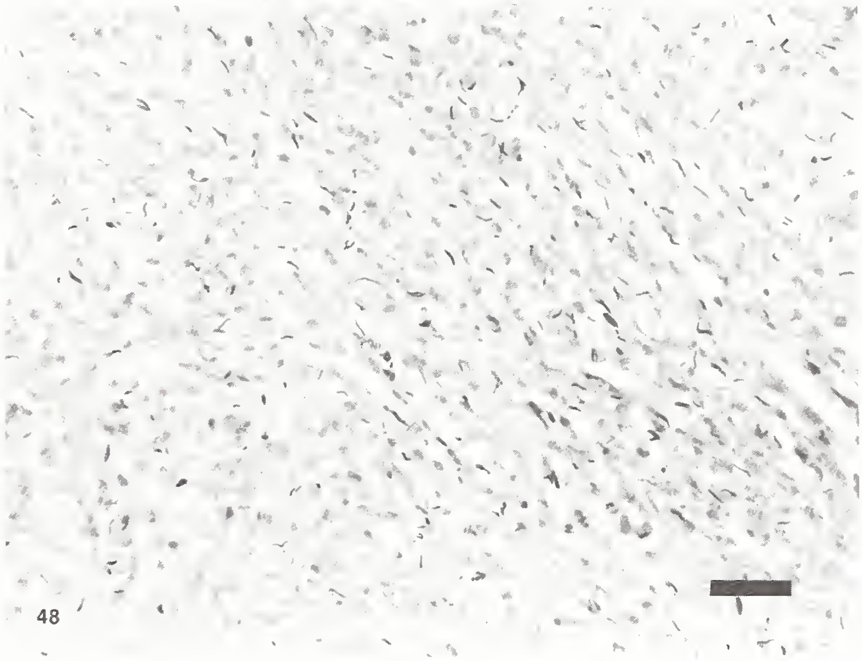

FIGURE 48.—Chronic inflammation (RTLA 420). Underexposed to show acid-fast, rod-shaped bacteria. Bar=30 μm.

especially acidophils may be present. Phagocytes form an epithelioid capsule around the active site and are in turn encapsulated by fibrous tissue (fig. 4). Because the active site is made anoxic, the destruction, exhaustion, or neutralization of toxins, invading microbes, normal tissues, phagocytes, etc., is facilitated.

In vertebrates, the mass of proliferating fibrobasts with accompanying capillary buds in the capsule is known as granulation tissue, and the lesion is known as a granuloma. Comparable, focal, chronic inflammatory reactions appear similarly in invertebrates but may be simpler because capillaries are absent and certain flattened hemocytes substitute for fibroblasts. Some people prefer the term "encapsulation reaction" for these invertebrate lesions but this seems incongruous. especially because the term "granuloma" is a misnomer (*43*). Granulomas have many variations and, although this description is oversimplified, it identifies a group of non-neoplastic lesions that may grossly appear tumor-like and microscopically have proliferating cellular components.

CONCLUDING REMARKS

A spectrum of borderline and non-neoplastic cases selected from the RTLA collection demonstrates the difficulty investigators have in distinguishing neoplasms from non-neoplastic lesions at one or more levels. At the gross morphology level, diagnosis is unreliable because, as shown in the examples, both types of lesions overlap in every visible trait, including size, color, texture, location, etc. The histopathology level is the one at which all cases should be studied because neoplasms are characterized by altered cellular morphology and behavior. In contrast, most of the pseudoneoplasms presented here represent variations in host response to a broad range of extracellular stress.

The examples given neither represent a complete list nor imply errors by contributors. The cooperation of the contributors in donating the cases and permitting their use is acknowledged with appreciation. Undoubtedly, not everyone will agree with all the conclusions, but perhaps this presentation will increase the awareness of problems in interpretation and encourage more thorough study at the histopathology level.

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(*42*) Dawe CJ: Some comparative morphological aspects of renal neoplasms in *Rana pipiens* and of lymphosarcomas in amphibia. *In* Biology of Amphibian Tumors (Mizell M, ed). New York, Heidelberg: Springer-Verlag, 1969, pp 429–440

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DISCUSSION

J. D. Hendricks: Dr. Harshbarger, I want to comment relative to your teratoid anomalies. We quite frequently see Siamese twinning in our rainbow trout, and often this will not become apparent until later in life, when you can see the Siamese attached to the normal fish. In one case in particular, in which these fish had been exposed to aflatoxin as embryos, the Siamese twin had a functional liver which was present just within the body cavity of the larger fish; we were able to produce tumors in both livers.

Hyperplastic Thyroid Lesions in Fish[1]

Karen L. Hoover[2, 3]

ABSTRACT—The teleost thyroid lacks a capsule, can be found ectopically in certain tissues throughout the animal, and is frequently predisposed to extensive proliferation. Several genetically susceptible small fish species as well as several larger salmonid and cyprinid species undergo a marked proliferation of the thyroid due to external conditions, such as poor water quality and abnormal nutrition, often making it difficult for one to distinguish these lesions as either cancer or goiter. The history of this controversy and present data from my study in which a marked growth of the thyroid occurred in guppies (*Poecilia reticulatus*) held at 27° C but not in those held at 17° C are reviewed. The significance of this predisposition in small fish to carcinogenicity testing is discussed. — Natl Cancer Inst Monogr 65: 275–289, 1984.

Beginning as early as the first decade of this century, the histological interpretation of the striking proliferative lesions observed in the thyroid of more than 30 fish species has been controversial (*1*). Although this growth was first reported by Bonnet (*2*) in lake trout in a German hatchery in 1883, its origin in the thyroid gland was not determined until 1902 by Plehn (*3*) who, with Pick (*4*), diagnosed the lesion as thyroid carcinoma. An extensive investigation of the disease took place in the United States following the deaths of thousands of brook trout in three New York State hatcheries in 1908. Between 1908 and 1914, Gaylord and his colleagues (*5*) intensively studied the biological behavior and possible causes of the enlarged masses protruding beneath the jaw, between the gills, and from under the operculum; they too were convinced that these growths were true carcinomas. However, Marine and Lenhart (*6–8*), following their study of affected brook trout in a private Pennsylvania hatchery, concluded that these masses were simply goiters. Numerous subsequent descriptions of similar proliferative lesions have followed, including those in wild salmonids from the Great Lakes (*9, 10*), marine fish in captivity (*11, 12*), and several species of small aquarium fish (*13–20*). Also since 1962, the Registry of Tumors in Lower Animals (located at the Smithsonian Institution, Washington, D.C.) has accessioned a total of 56 specimens of thyroid growths from 24 fish species representing 2 families of Chondrichthyes and 4 families of Osteichthyes; such lesions have been diagnosed as either goiters or tumors.

Neoplasms characteristically exhibit autonomous growth which may be invasive locally and ultimately metastasize to distant sites. Tumors frequently occur in a higher incidence as the animal ages and will continue to grow when transplanted from the original host to a new syngeneic recipient. Some or all of these features are common to thyroid growths in particular fish species, but five unique characteristics of this tissue in several fish species studied confound the diagnosis of these lesions, as two support and three argue against their neoplastic nature.

Firstly, the thyroid is an unencapsulated tissue in teleosts, appearing as a few, variably sized colloid-filled follicles along the ventral aorta which runs beneath the bony and cartilaginous bridge uniting the opposing gill arches and forming the floor of the mouth. Follicles are most numerous within the connective tissue adjacent to the first and second branchial arteries as they split off the ventral aorta. Less commonly, follicles may extend back to the most caudal fourth gill arch. Adjacent muscle, cartilage, and even the medullary spaces of bone may also contain random follicles (*21*). As described by Gaylord and Marsh (*5*) in the trout, developmental events during embryonic and juvenile life account for this distribution. Following the evagination and separation of a portion of the ventral pharyngeal epithelium, the hollow sphere of precursor cells progressively elongates and grows out from the bifurcation of the heart tube, ventral to the aorta, where it lies after separation from the pharynx. Multiplying buds of thyroid epithelium grow around the aorta toward the dorsal surface and follow its anterior course toward the branchial chamber. By day 77 in the juvenile trout, thyroid follicles are scattered along the dorsal aspect of the ventral aorta from the bifurcation of the heart tube at the bulbus arteriosus to the first and second branchial arteries (*5*). This distribution varies according to the species. For example, in the platyfish, follicles normally lie along the inferior jugular vein lying dorsal to the heart as well.

Secondly, according to Baker (*16*), the thyroid follicles in some species can continue to multiply and then migrate along the surface of the inferior jugular to the ophthalmic

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ABBREVIATIONS: *N*-OH-FAA = *N*-hydroxy-fluorenylacetamide; H & E = hematoxylin and eosin.

[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Nutrition and Metabolism Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute (NCI), Frederick Cancer Research Facility, Building 538, Room 205-C, Frederick, Maryland 21701.

[3] I thank Dr. Marty Wenk and Mr. John Dean of Microbiological Associates, Bethesda, Maryland; Mrs. Barbara Coolidge and Mrs. Ruby Thompson of the NCI for their scientific and technical assistance; Mrs. Kevin Beall and Mrs. Marcha Gamber of the NCI, Frederick, Maryland, for secretarial assistance; and Larry Ostby and Gary Best, NCI, for the photography.

vein and into the optic choroid or along the cardinal veins to the kidney and along distant vasculature, forming ectopic deposits in the kidney, spleen, intestine, swim bladder, liver, and the heart. This explanation by Baker followed extensive observations of the sequential thyroid behavior in the BH strain of platyfish, a small aquarium fish particularly prone to the development of thyroid growths during experiments. They also found a correlation between increased proliferative activity of the pharyngeal thyroid tissue with age and the presence of ectopic tissue. Such ectopic foci of otherwise normal tissue within a fish exhibiting marked pharyngeal thyroid hyperplasia could then logically be construed as metastases. Their experimental observations supporting the migratory and physiologic behavior of thyroid are summarized in table 1. Ectopic thyroid has been widely reported in several fish species (including guppies, goldfish, swordtails, trout, and barbs) as well as in mammals, including man (*16*).

Thirdly, the increase in thyroid growth incidence is age related in 4 small fish species which are prone to development of these growths, i.e., the guppy, Amazon molly, swordtail, and the BH strain of platyfish, with a 30 to nearly 100% incidence in each species by 2–3 years. By contrast, thyroid growths in the trout can appear at 6 months of age and usually are most common in 2- and 3-year old fish before regressing in older adults. Marine and Lenhart (*6*) considered this further proof of their goitrous nature in trout because this age-related frequency is found among humans suffering from goiter.

Fourthly, Hart and Setlow (*22*) reported the subsequent growth of large thyroid tumors in syngeneic mollies receiving UV- or gamma-irradiated normal thyroid cells from normal donors. Although not derived from established tumors at the time of inoculation, these cells were presumptively neoplastic, and the investigators' observations suggested the formation of thyroid tumors from them. Such studies indirectly indicate the transplantability of neoplastic thyroid cells. By contrast, Marine and Lenhart (*6*) found no successful transplants of thyroid tissue implanted subcutaneously in 15 recipient brook trout.

Lastly, thyroid growth can regress in fish after the addition of potassium iodide to the water, an improvement in the general water quality, or a change in the diet. Upon exposure of affected trout (aged 20–41 mo) to potassium iodide in the water for 10–62 days, Marine and Lenhart (*6, 7*) reported that the actively proliferated thyroid epithelium reverted to a resting state in which colloid-filled follicles lined by low cuboidal epithelium predominated, with an increased amount of connective tissue frequently present. They also observed similar changes among fish when their diet had been changed from beef liver to sea fish and among trout living in streams accessible to abundant fresh clean water following their removal from crowded ponds (*8*). The involution of the thyroid, although noted by Marine and Lenhart to have taken place to varying degrees in different fish, almost always occurred regardless of age and extent of thyroid hyperplasia. These observations were the basis of their contention that the growths were simple hyperplasias. Gaylord and Marsh (*5*), noting similar involuting changes after treatment of affected fish with arsenic pentoxide, mercuric chloride, or potassium iodide in concentrations as low as 1:3,000,000 to 1:32,000,000, maintained that this response supported the diagnosis of carcinoma, inasmuch as therapy with heavy metals had been effective in the treatment of some neoplasms in experimental animals and humans. They pointed out the probable necrogenic effect of heavy metals on the tumor vasculature because hemorrhagic areas were commonly found in regressing tumors. However, from their description of regressing fish thyroid tumors following exposure to the various metals, only arsenic and mercury were associated with hemorrhagic changes, which suggested a different mode of action for the therapeutic effect of iodine.

TABLE 1.—*Experimental observations in the BH strain platyfish* (Xiphophorus)[a]

1) Platys older than 7 mo: progressive distribution of isolated follicles along cardinal veins, surface of kidney,
2) Close correlation between pharyngeal thyroid tissue and increased incidence of ectopic thyroid,
3) Young platys in potassium iodide-treated water: low incidence of both hyperplastic pharyngeal thyroid and ecotopic thyroid,
4) Older platys in potassium iodide-treated water: regression of pharyngeal and ectopic thyroid growths,
5) Prolonged treatment of the goitrogen thiourea in young platys: proliferation of pharyngeal and ectopic thyroid, and
6) No development of ectopic tissue in platys thyroidectomized early in life by uptake of ^{131}I

[a] *See* (*16, 17*).

TABLE 2.—*Proliferation of fish thyroid: Suggested factors*

Factor	References
Iodine deficiency	(*5, 7, 15*)
Poor water quality	(*9, 11, 12*)
Low O_2 tension	(*11*)
Salinity changes	(*11*)
Abnormal calcium:magnesium ratios	(*11*)
Water pollution	
Accumulating metabolites (urea, ammonia, etc.)	(*7, 11*)
Organochlorines	(*9*)
Genetics	(*13, 14, 15–18, 19, 20*)

It is apparent from numerous reports that multiple extrinsic and intrinsic factors probably provoke thyroid hyperplasia, either by increasing the physiological need for iodine or by directly stimulating thyroid follicular proliferation independent of iodine levels. The suggested causes are listed in table 2. Thyroid masses occasionally have been reported in marine, usually captive, fish [*see* (*12*)] that would indicate the presence of iodine in the water does not necessarily prevent follicular hyperplasia. On the other hand, some investigators (*23–25*) suggest certain species have a higher metabolic need for iodine that may not be satisfied by existing levels in a marine culture or even a natural environment. In fact, analyses of iodine levels in the saltwater at the Philadelphia (*13*) and New York aquaria (*24*) revealed lower levels than those normally found in the oceans (~8–20 g/liter vs. up to 50 g/liter in ocean water). Moreover, Pickford (*26*) found that dietary supplements of iodine were required to prevent thyroid hyperplasia in *Fundulus*, a cyprinodont reared in saltwater.

DEVELOPMENT OF THYROID GROWTHS

In their classic work, Gaylord and Marsh (*5*) described and included numerous excellent sketches and photographs of the gross and microscopic genesis of these lesions in the salmonid. The first clinical sign is a diffuse reddening of the floor of the mouth eventually followed by small discrete nodules protruding through this area. Ultimately, large nodular masses may emerge from beneath the operculum and below the jaw as the thyroid progressively enlarges laterally and ventrally. Histologically, individual cells comprising the follicles which are normally lined by cuboidal or columnar cells undergo hypertrophy followed by proliferation and formation of secondary variably shaped and sized follicles. These proliferated cells have a deeply basophilic cytoplasm and appear squamous, cuboidal, or columnar. Increased capillary growth into the area (neovascularization) accounts for the reddish color of the lesions. Sometimes, the follicles are cystic and may contain a papillary infolding of the lining epithelium. Less frequently, the tumor can assume a solid appearance exhibiting closely packed, sometimes swollen, follicular cells or a glandular morphology with alveoli filled with follicular cells or microfollicles. Rarely, the epithelial cells assume a spindle shape and appear almost sarcomatous. The infiltration of adjacent muscle fibers by follicles as well as the destruction of adjoining bone and cartilage and their active spread anteroposteriorally along the pharynx, around the heart, and laterally toward the distal portions of the gills can occur. However, Gorbman and Gordon (*13*) noted the progressive dissection of muscle fibers as follicles proliferated along the endomysium and apparently were not directly destroying the muscle fibers. On the other hand, they did observe the destruction of bone and cartilage in the swordtail. Despite this apparent active invasiveness by the proliferating thyroid, Baker-Cohen (*27*) concluded that ". . . invasiveness may be regarded as a natural characteristic of thyroid tissue in the normal as well as pathologic condition. . . . these tumors should be classed as goiterous growths even though they were lethal." When vascular invasion has been observed, however, the growth should certainly be regarded as carcinomatous because such behavior is a true feature of malignancy, and normal thyroid has not been observed in intramural sites of blood vessels. Gaylord and Marsh (*5*) noted one such case of vascular invasion in a trout.

MATERIALS AND METHODS

During a study assessing the comparative effects of the carcinogen *N*-hydroxy-fluorenylacetamide on female guppies held at different temperatures (17° and 27° C), I observed raised pink to reddish growths protruding from beneath the jaws of many of those fish held at 27° C, regardless of treatment.

TABLE 3.—*Composition of trout diet fed to guppies*[a]

Ingredient	Percent
Fish meal	≥45
Soy flour	15
Dried whey	5
Dried yeast	5
Dried blood flour	5
Fish solubles	5
Trace mineral mix	1
Soybean oil	10
Vitamin premix	0.4
Choline chloride	0.1
Ethoxyquin	150 ppm

[a] Diet was formulated by Glenco Mills, Glenco, Minnesota.

Initially, 3-week old female guppies were divided among 6 aquaria (30-gallon) in groups of 80/tank. They were fed either the control diet, which was a trout feed formulated by Glenco Mills (Glenco, Minn.) or the same diet impregnated with either 0.04% or 0.08% *N*-hydroxy-fluorenylacetamide (table 3). Animals at each treatment were held at either 17° or 27° C. Deionized water with sodium bicarbonate, calcium sulfate, magnesium sulfate, and potassium chloride added was used in all aquaria. The total hardness of the water was maintained at 150–200 ppm. Seven months later, 116 guppies with nutritional problems that led to fatty and fibrotic changes in the liver were killed. A large number of mortalities had occurred among the fish in all tanks held at 27° C. The visceral block (including the intestine, liver, ovary, spleen, and rarely anterior or posterior kidney, or both) and the remaining body cavity (containing the pharyngeal area, heart, kidney, eye, swim bladder, and occasionally, the gonad) were fixed in Bouin's fixative for 48 hours. The tissues were subsequently rinsed twice in 70% ethanol before they were embedded for histological examination.

RESULTS

As summarized in table 4, most fish held at 27° C frequently had extensive pharyngeal thyroid growths which protruded beneath the jaw, extending laterally into the gill lamellae and caudally toward and around the heart in some fish (figs. 1–3). In 1 fish, a few follicles lay within the ventricular myocardium as well. In addition, 13 guppies held at this temperature had well-circumscribed masses of thyroid tissue which usually lay within the mesentery adjacent to the liver in the sections of visceral block. Almost always, the proliferation was so extensive that it was difficult for me to recognize the original organ site. The tissue infiltrated was considered to be the spleen because of

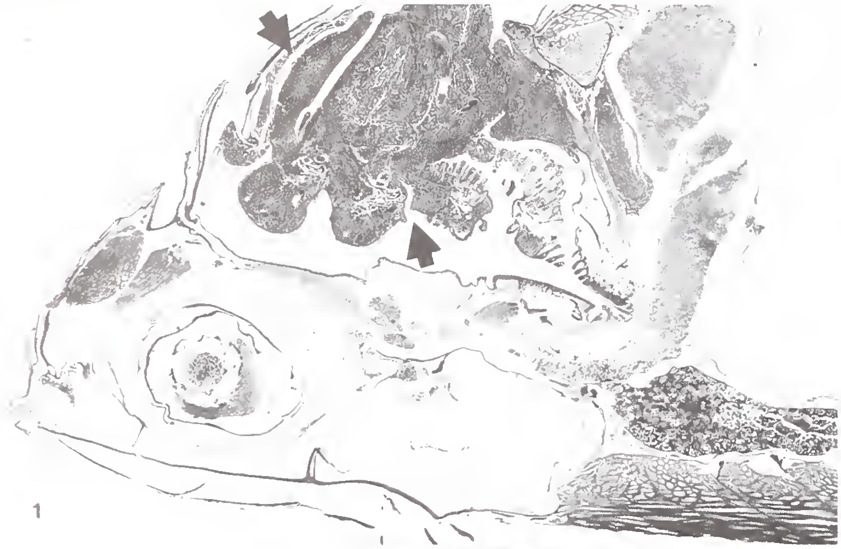


FIGURE 1.—Hyperplastic thyroid involving extensive area throughout pharyngeal site. Treatment: 27° C and 0.04% *N*-OH-FAA. H & E. × 14

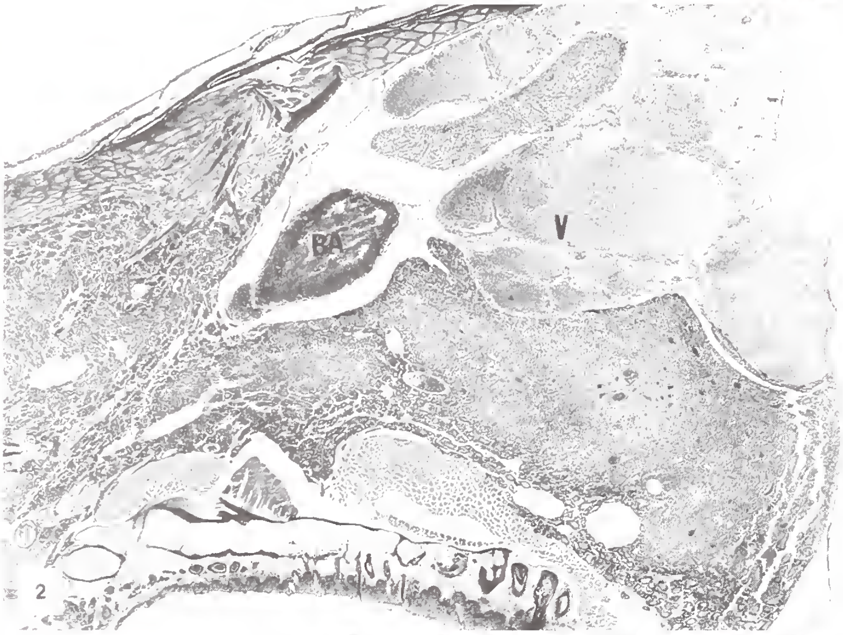


FIGURE 2.—Hyperplastic thyroid extending caudally from pharyngeal area, surrounding bulbus arteriosus (BA) and impinging on ventricle (V) of heart. Thyroid cells within growth assume a glandular as well as solid architecture. Treatment: 27° C and 0.04% *N*-OH-FAA. H & E. × 54

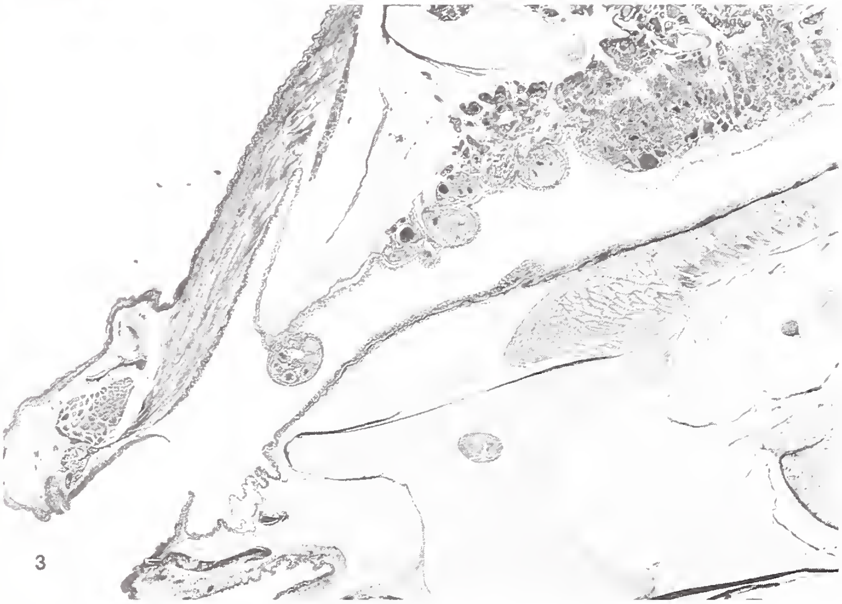


FIGURE 3.—Exophytic protrusions of hyperplastic thyroid along pharyngeal surface. Treatment: 27° C and 0.04% *N*-OH-FAA. H & E. × 38

TABLE 4.—*Incidence of hyperplastic and ectopic thyroid in control and* N-*OH-FAA-treated guppies held at 2 temperatures*

Temperature, degrees C[a]	Percent *N*-OH-FAA[b]	No. affected fish/total No. fish containing target tissue in section							
		Hyperplastic thyroid	Per-cent	Splenic thyroid	Per-cent	Extra-pharyngeal thyroid[c]	Per-cent	Total with ectopic tissue	Per-cent
27	0	11/12	92	4/6	67	3/12	25	4/12	33
17	0	0/16	0	0/10	0	1/16	6	1/16	6
27	0.04	10/12	83	5/8	62	5/12	42	5/12	42
17	0.04	3/32	9	0/14	0	0/32	0	0/32	0
27	0.08	11/12	92	4/8	50	1/12	8	4/12	33
17	0.08	2/32	6	0/32	0	0/32	0	0/32	0

[a] Guppies were maintained at temperatures indicated for 7 mo.

[b] Either of the concentrations of *N*-OH-FAA was added to the diet for 6 mo.

[c] Values include sites in the pancreas, liver, optic choroid, mesenteric fat, anterior kidney, ovary, and intestine. Each affected fish was counted only once.

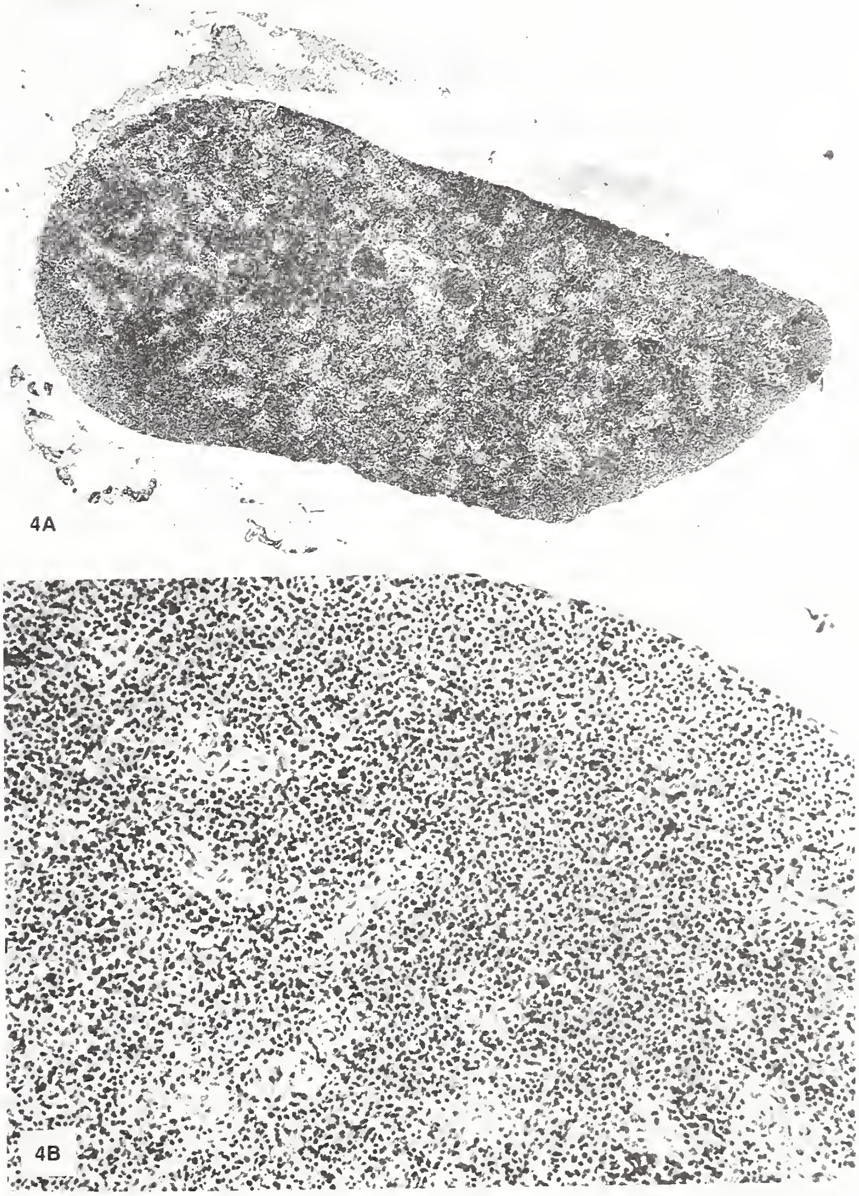
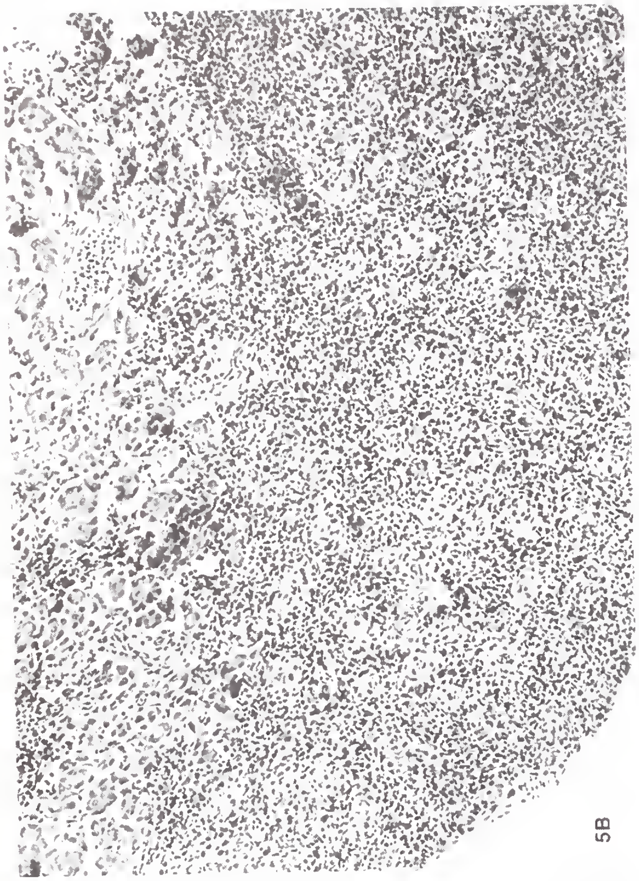


FIGURE 4.—A) Normal spleen. *Note* large sinuses filled with nucleated red blood cells and macrophage-melanin centers (*arrows*). Treatment: 17° C and 0.08% *N*-OH-FAA. H & E. × 80. B) Normal spleen. Higher magnification showing relationship of connective tissue trabeculae and vascular sinuses. Treatment: 17° C and 0.08% *N*-OH-FAA. H & E. × 330



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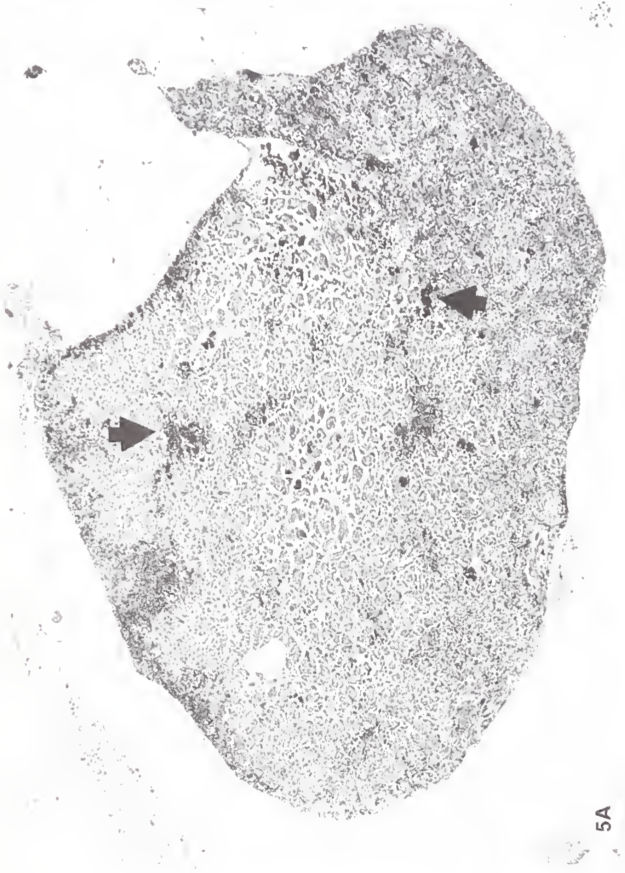

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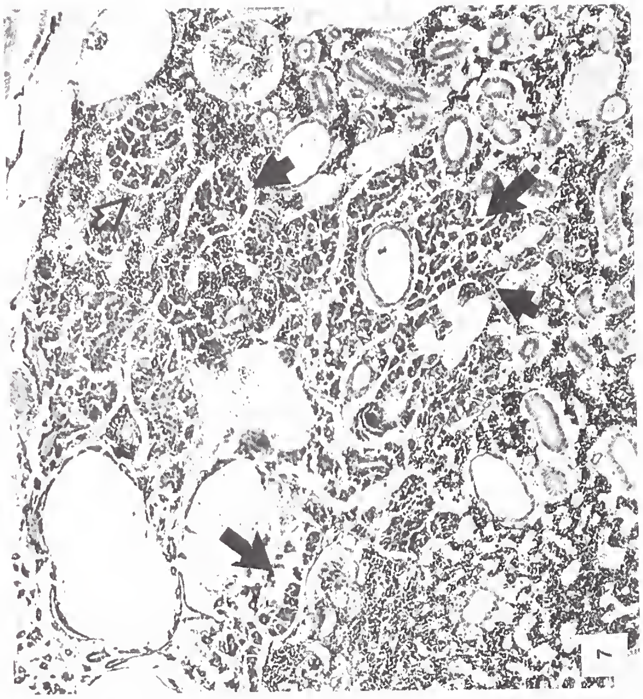

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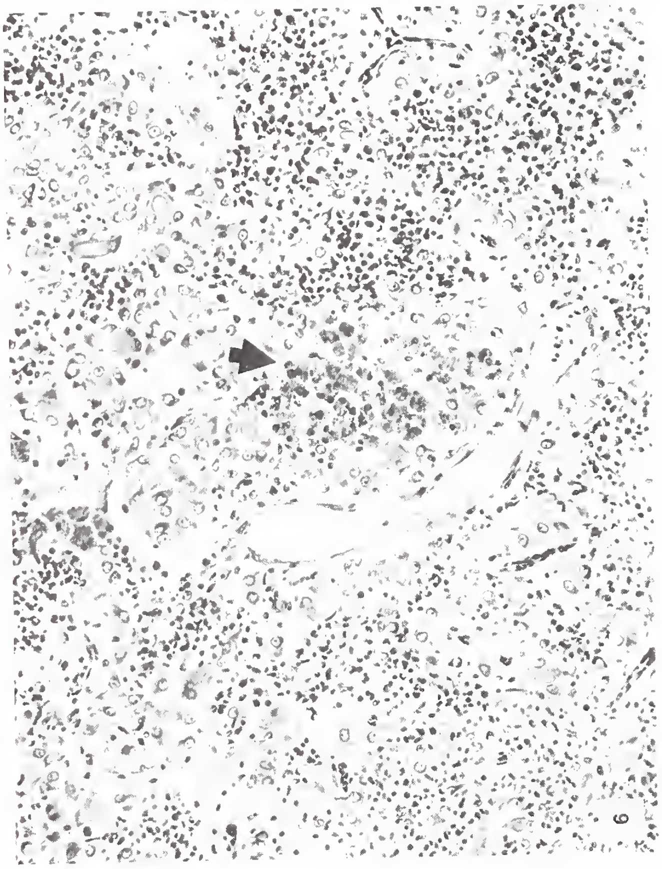

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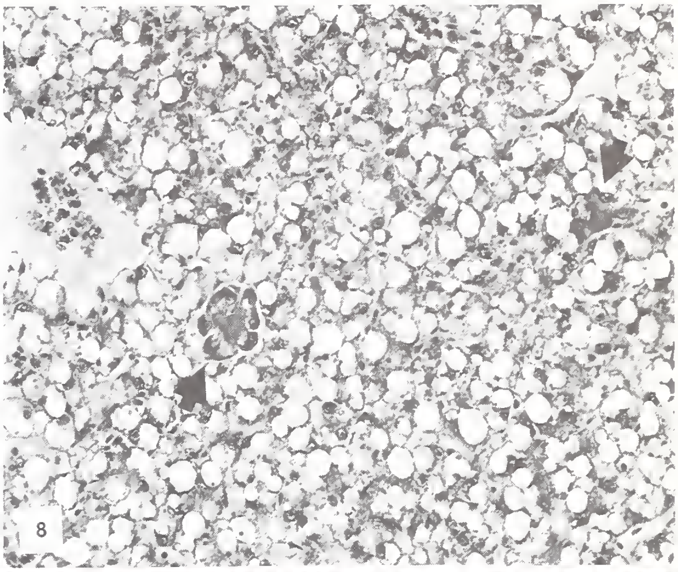
1) its location on the tissue block; 2) the presence of lymphoid cells, blood sinuses, and connective tissue trabeculae at the periphery of the growths; 3) the presence of macrophage–melanin centers within the infiltrates that are normally found in the spleens and livers of fish; and 4) the usual inclusion of the spleens in the absence of the kidney from the visceral block (figs. 4–6). Moreover, thyroid tissue was observed in only 1 anterior kidney examined within a body cavity section (fig. 7). In addition to the spleen, heart, and anterior kidney, thyroid follicles were observed in the liver, pancreas, and choroid gland of each of 3 fish and in the mesenteric fat, intestine, and ovary of 1 fish at each tissue site (figs. 8–11, table 4). The histological appearances of the pharyngeal and extrapharyngeal thyroid conformed to previous descriptions of the tissue in salmonids and other small aquarium fish. In general, follicular cells assumed a cuboidal, columnar, or plump and slightly elongated shape, forming follicles, tubules, or solid sheets of cells (figs. 12–14). The cytoplasm was often deeply basophilic and the cells contained a centrally placed large round nucleus with a prominent nucleolus; the nucleus lay adjacent to a clear area facing the follicular lumen (fig. 8). Occasionally, the cytoplasm appeared eosinophilic and globular (fig. 15), and the eosinophilic colloid within the follicles assumed a solid, vacuolated, or granular appearance (fig. 16). Rarely, the thyroid cells were elongated and arranged in a solid pattern (fig. 17). Both pharyngeal and extrapharyngeal thyroid sometimes contained cystic areas with flattened follicular epithelium lining large empty or colloid-filled lumina (fig. 18). In the pharyngeal area, proliferated thyroid infiltrated between muscle fibers (fig. 19) and, in a few fish, a few follicles lay within the cartilaginous framework in the area (fig. 20). Stromal tissue was generally scanty, and mitotic figures were infrequently observed.

Although in almost all fish, evidence of active invasion was absent, a few exhibited local degeneration of muscle fibers adjacent to proliferated thyroid follicles in the pharyngeal area (fig. 19); thyroid follicles lay within a blood vessel in the spleen in 1 fish (fig. 21), and a collection of follicles lay immediately outside the capsule of a spleen filled with thyroid follicles, which suggested capsular invasion (fig. 22). However, it cannot be ruled out that the latter 2 observations could be attributable to the accidental displacement of the thyroid tissue during histological processing of the sections in question.



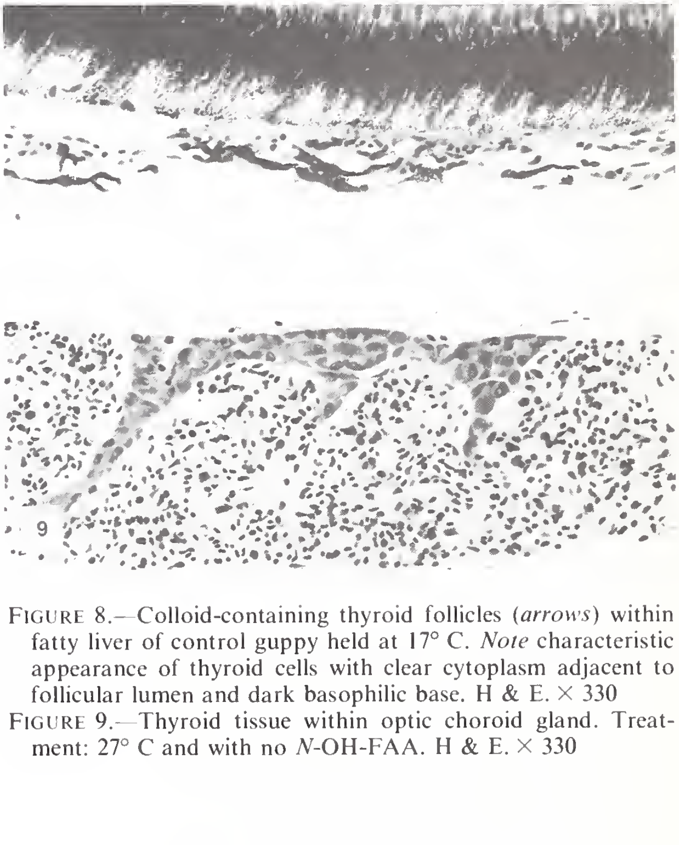


FIGURE 8.—Colloid-containing thyroid follicles (*arrows*) within fatty liver of control guppy held at 17° C. *Note* characteristic appearance of thyroid cells with clear cytoplasm adjacent to follicular lumen and dark basophilic base. H & E. × 330

FIGURE 9.—Thyroid tissue within optic choroid gland. Treatment: 27° C and with no *N*-OH-FAA. H & E. × 330

←

FIGURE 5.—A) Well-circumscribed mass in mesentery containing large area of thyroid cells. Vascular sinuses evident at lower right and collections of lymphoid cells and macrophage-melanin centers (*arrows*) throughout tissue strongly suggest affected site as spleen. Treatment: 27° C and with no *N*-OH-FAA. H & E. × 80. B) Higher magnification of thyroid tissue in mesenteric mass, showing adjacent vascular sinuses characteristic of spleen. Treatment: 27° C and with no *N*-OH-FAA. H & E. × 330

FIGURE 6.—Higher magnification of thyroid tissue in mesenteric mass showing macrophage-melanin center, usually found in the spleen. Treatment: 27° C and with no *N*-OH-FAA. H & E. × 330

FIGURE 7.—Thyroid tissue in anterior kidney (*arrows*). Treatment: 27° C and with no *N*-OH-FAA. H & E. × 80

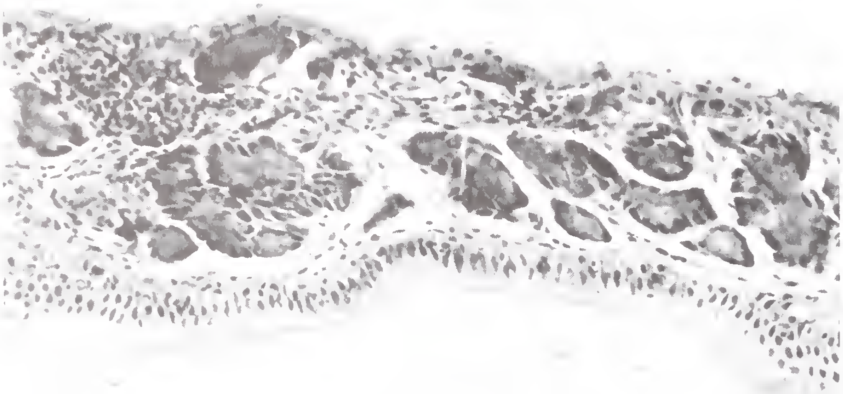


FIGURE 10.—Thyroid follicles within intestinal wall. Treatment: 27° C and 0.04% *N*-OH-FAA. H & E. × 330

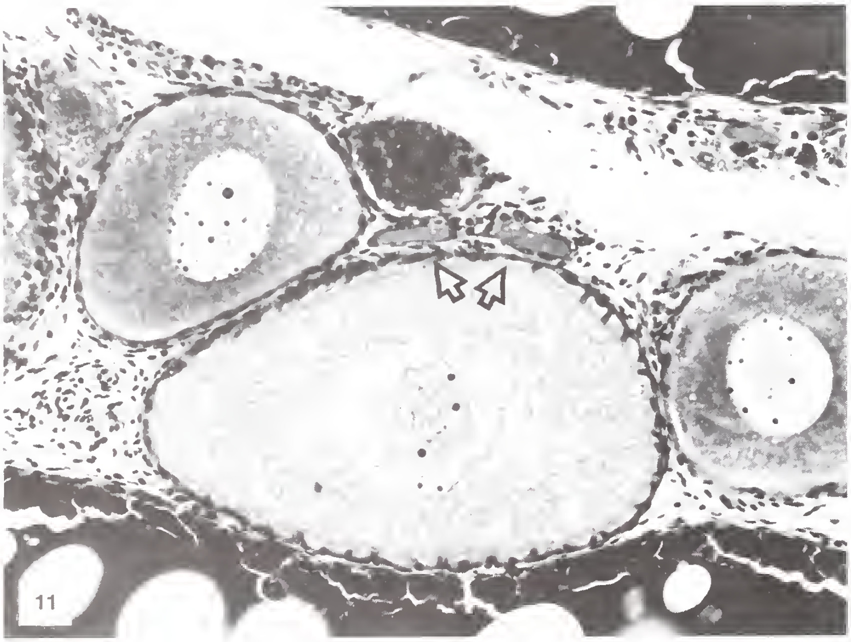


FIGURE 11.—Thyroid follicles within ovary. Treatment: 27° C and with no *N*-OH-FAA. H & E. × 330

By contrast, the fish held at 17° C did not show ectopic thyroid except in 1 untreated fish in which follicles lay within the liver (fig. 8). Pharyngeal thyroid proliferation was only infrequently observed and was not as extensive as in the fish held at 27° C. When examined in body cavity sections, thyroid follicles in these fish lay randomly along the ventral aorta (fig. 23).

DISCUSSION

The results of this study demonstrated a striking dichotomy in the extent of thyroid follicular cell growth between guppies held at a warm temperature (27° C) and those held in a cooler temperature (17° C). The close correlation between extensive thyroid hyperplasia in the pharyngeal area and the presence of thyroid tissue in ectopic sites suggests the physiological similarity of thyroid at the different tissue sites responding in a hyperplastic fashion to physiological or environmental factors, or both. These findings support the conclusion Baker (*17*) drew from her observations on iodine and thiourea-treated platyfish, i.e., that follicular cell response, involution and hyperplasia, respectively, were parallelled in pharyngeal and extrapharyngeal thyroid sites. Likewise, animals living in iodine-poor water exhibited hyperplasia of follicular cells in both pharyngeal and ectopic tissue sites. In goldfish, Chavin and Bouwman (*28*) found similar proportions of the iodinated thyronine intermediates and thyroxine in both pharyngeal and renal thyroid that further confirmed the analogous relationship.

In my study, one or more environmental factors could have initiated the physiological reactions leading to thyroid hyperplasia, whether indirectly through the pituitary–thyroid axis or directly on the thyroid tissue itself. Iodine

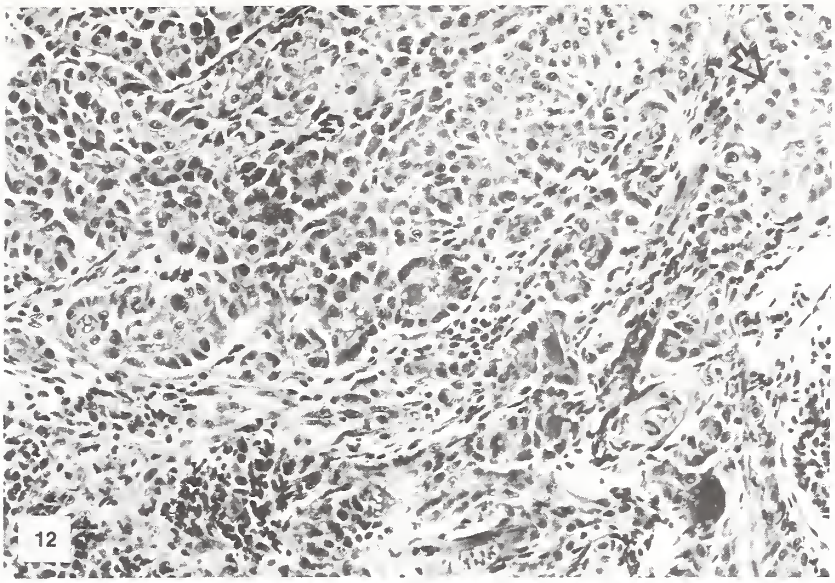


FIGURE 12.—Follicular thyroid cells within pharynx, assuming a cuboidal appearance. *Note* swollen follicular cells in *upper right corner* (*arrow*) and vacuolated colloid within some follicular lumina. Treatment: 27° C and 0.04% *N*-OH-FAA. H & E. × 330

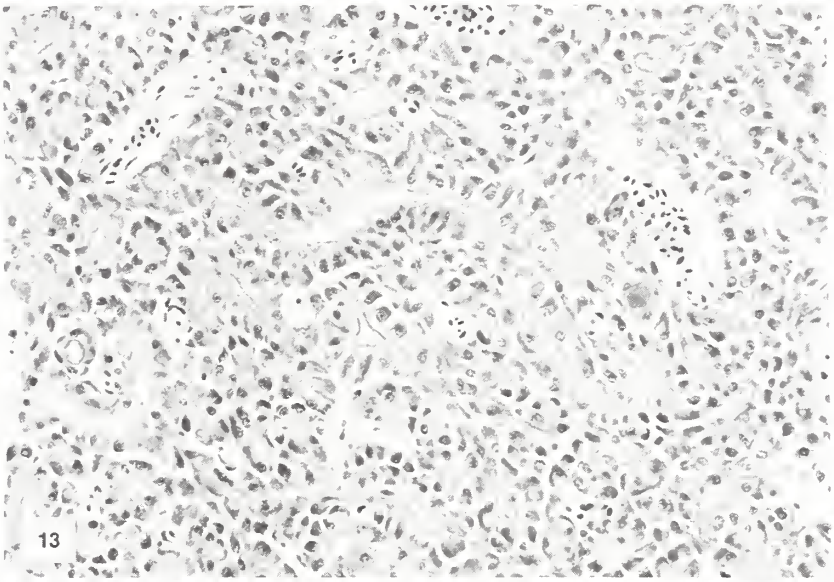


FIGURE 13.—Hyperplastic pharyngeal thyroid assuming a glandular architecture. Treatment: 27° C and with no *N*-OH-FAA. H & E. × 330

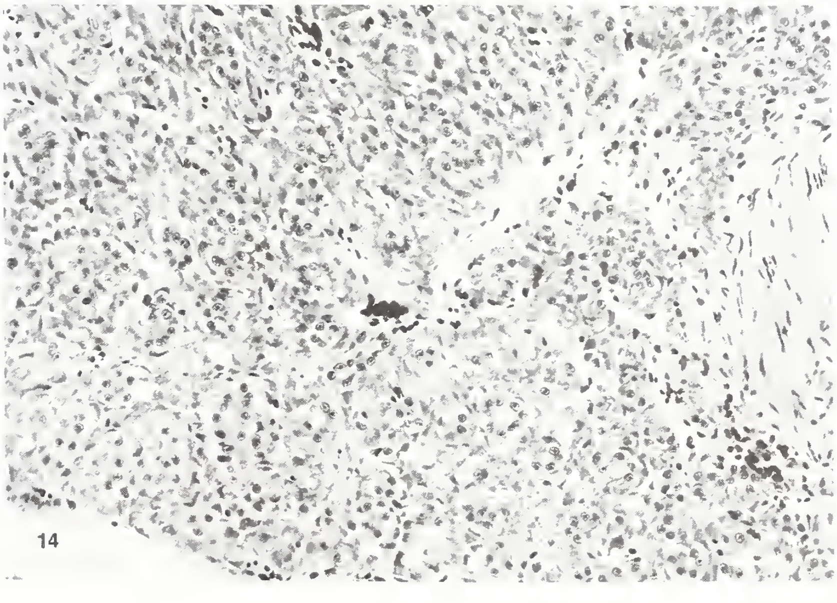
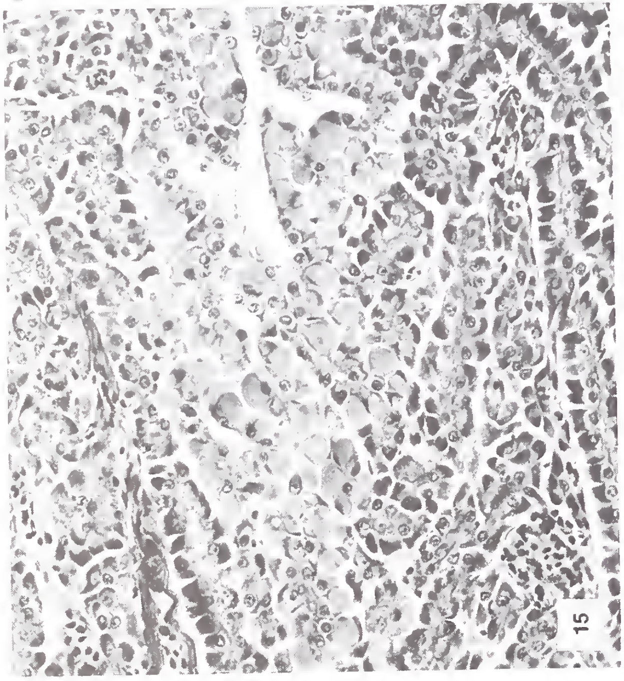


FIGURE 14.—Solid pattern of closely packed follicular cells within hyperplastic pharyngeal thyroid. Treatment: 27° C and 0.04% *N*-OH-FAA. H & E. × 330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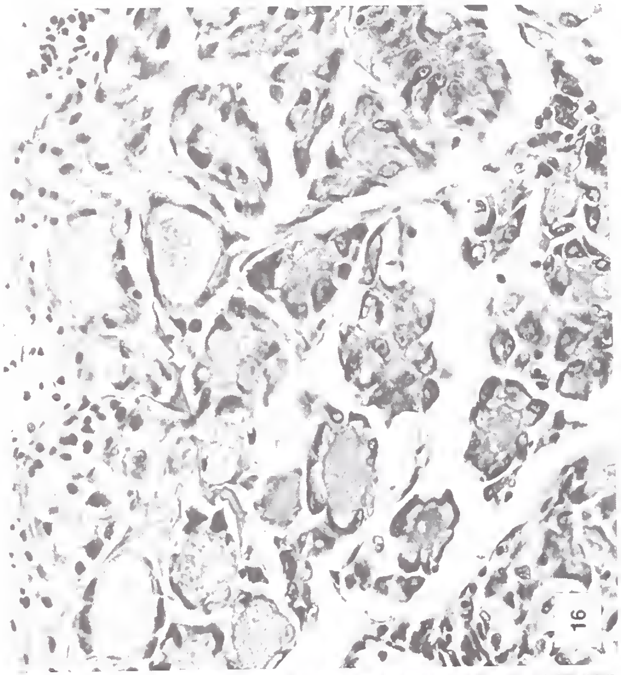
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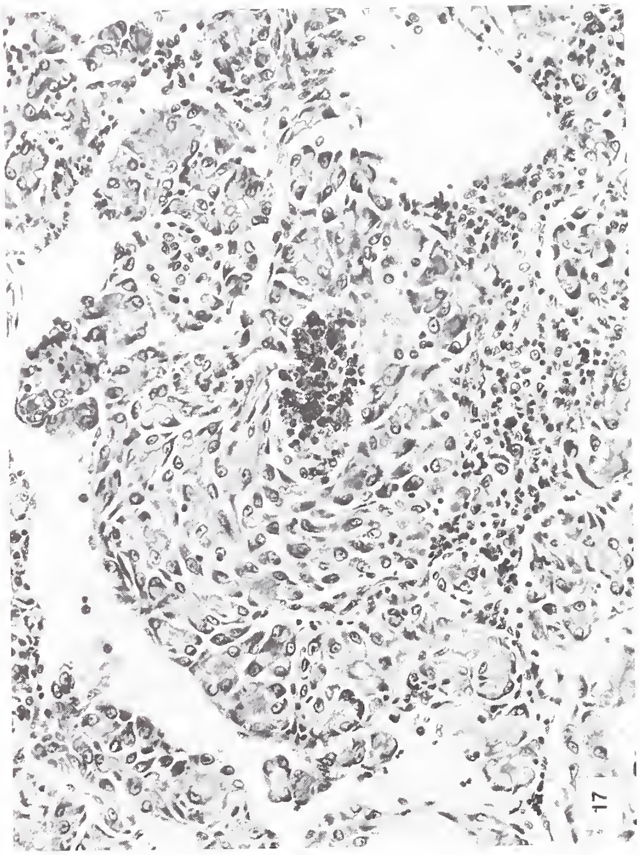
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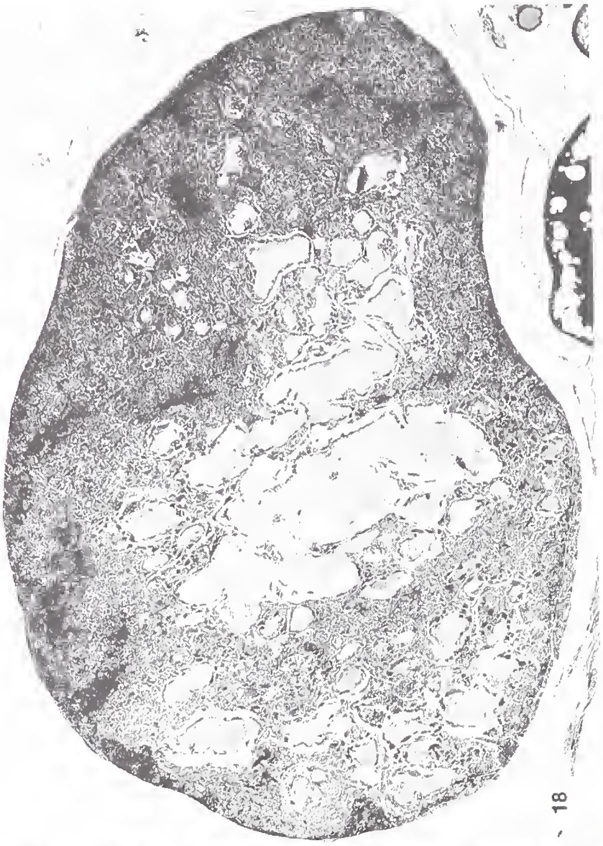
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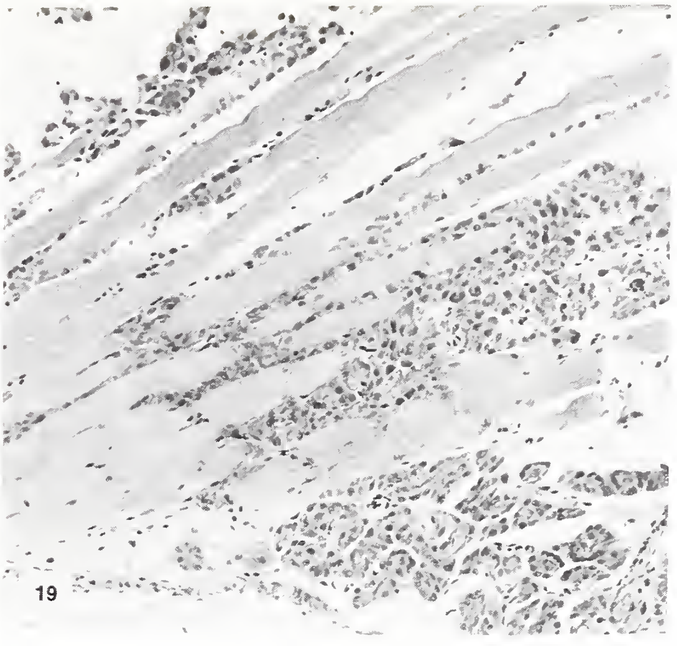

FIGURE 19.—Growth of thyroid follicles between muscle fibers within pharyngeal area. *Note* loss of striation and granular appearance of muscle undergoing degeneration. Treatment: 27° C and with no *N*-OH-FAA. H & E. × 220

deficiency is a major stress factor to be considered. Although the guppies were fed a diet which presumably contained iodine in the fish oil, no measurements of iodine levels in the diet were made nor was iodine added to the water. When we added 0.005% potassium iodide to the water in tanks containing fathead minnows (*Pimephales promelas*) with similarly appearing thyroid growths, the thyroid hyperplasia subsequently diminished, which suggested a similar result from iodine deficiency in affected guppies. Diminished thyroid activity and thus a decreased need for iodine could account for the normal or only mildly hyperplastic thyroids in the guppies maintained at 17° C. Cold ambient temperatures have inhibited thyroid activity in trout and the cyprinid *Phoxinus laevis*, as measured by radioiodine methods, the staining reaction of the colloid by azan blue, and the comparative size of follicular cell area to total follicular area (*29, 30*). Using the last technique, Fortune (*30, 31*) found that the minnow *Phoxinus*, but not the guppy, responded more rapidly to thiourea treatment with increased thyroid activity than did the fish held at colder temperatures. This observation suggested normally increased thyroid activity at the higher temperatures. He also reported morphological evidence of increased secretory activity in the pituitary thyrotrophs (vacuolation and reduced cell contents) and subsequent increased thyroid activity in *Phoxinus* held at 25° C, whereas those fish held below 10° C exhibited low activity at both sites. Thus in the present study, the existing iodine deficiency may have been aggravated by water and possibly diet of the metabolically more active guppies held at 27° C, a condition not exacerbated among the metabolically less active fish held at 17° C. Perhaps the cold environment, normal for these warmwater fish, inhibited thyrotroph activity in the pituitary (*31*) or neural and biochemical pathways of the pituitary-thyroid axis, thereby preventing a reactive hyperplasia due to iodine deficiency. Alternatively, Barrington and Matty (*32*) suggested that the sexual cycle of *Phoxinus* (specifically the estrogen levels in females) influences thyroid activity in an inverse manner. Thus the altered thyroid activity may be a consequence of a direct effect of temperature on other tissue sites, in this instance the gonad or pituitary gonadotrophs, or both. Inferior water quality might also be a factor as the water would be more quickly fouled by the greater output of urinary metabolites from the guppies held at 27° C. However, in my study, the nitrite, pH, oxygen, and hardness (calcium and magnesium) levels were regularly monitored in all tanks and were generally satisfactory; we routinely replaced one-third of the water in each tank every other week to prevent deterioration of water quality. These factors were probably not important in the genesis of the thyroid hyperplasia, nor was the fatty change in the livers likely associated with the thyroid condition, as fish in all tanks (warmwater and coldwater) exhibited this condition. Measuring the levels of the di-iodinated and tri-iodinated thyroid hormones in a study of the Great Lakes salmon, Moccia et al. (*10*) found higher serum levels of both in goiterous fish than in the euthyroid salmon, but they suggested that their physiological availability to the tissues may have differed between the 2 groups of fish, thereby initiating the hypothyroid condition. Such an explanation could also be considered here.

Hulbert (*33*), in discussing the effect of these hormones on mammals, hypothesized that the site of action may well be intracellular as well as on surface membranes of various tissues, thereby influencing events occurring at these sites dependent on enzyme- or receptor-site activity. Extracellular and intracellular membrane changes, resulting from the activity of an abnormal thyroid, conceivably could have profound effects on the manner in which fish as

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FIGURE 15.—Swollen eosinophilic thyroid cells assuming a "globular" appearance within hyperplastic pharyngeal thyroid. Treatment: 27° C and 0.04% *N*-OH-FAA. H & E. × 330

FIGURE 16.—Elongated thyroid follicular cells surrounding colloid with granular appearance. Treatment: 27° C and 0.04% *N*-OH-FAA. H & E. × 540

FIGURE 17.—Atypical appearance of thyroid follicular cells within mesenteric mass (presumably spleen). Cells are elongated and arranged in a solid pattern. Treatment: 27° C and 0.04% *N*-OH-FAA. H & E. × 330

FIGURE 18.—Cystic thyroid tissue within mesenteric mass (presumably spleen). Treatment: 27° C and 0.08% *N*-OH-FAA. H & E. × 54

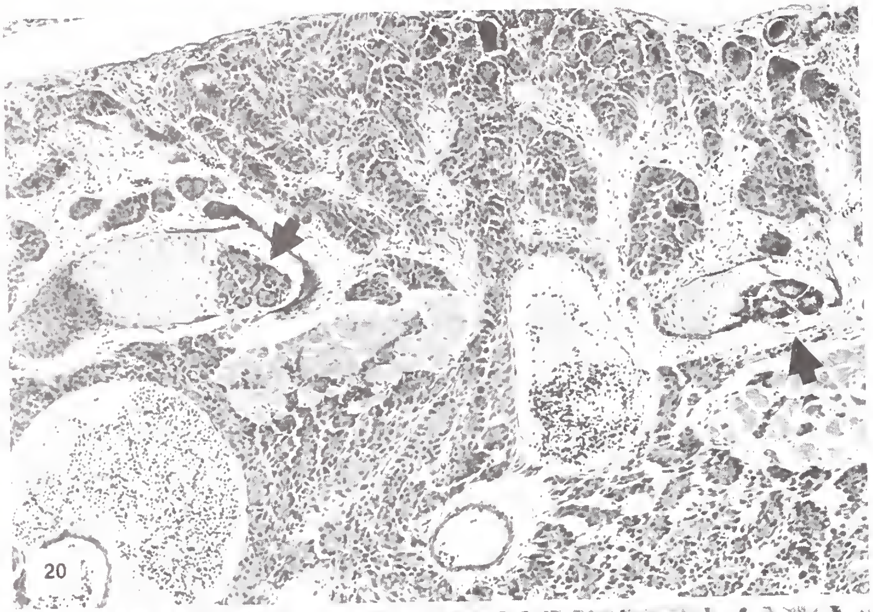

FIGURE 20.—Thyroid follicles within cartilaginous framework in pharyngeal area (*arrow*). Treatment: 27° C and 0.04% *N*-OH-FAA. H & E. × 130

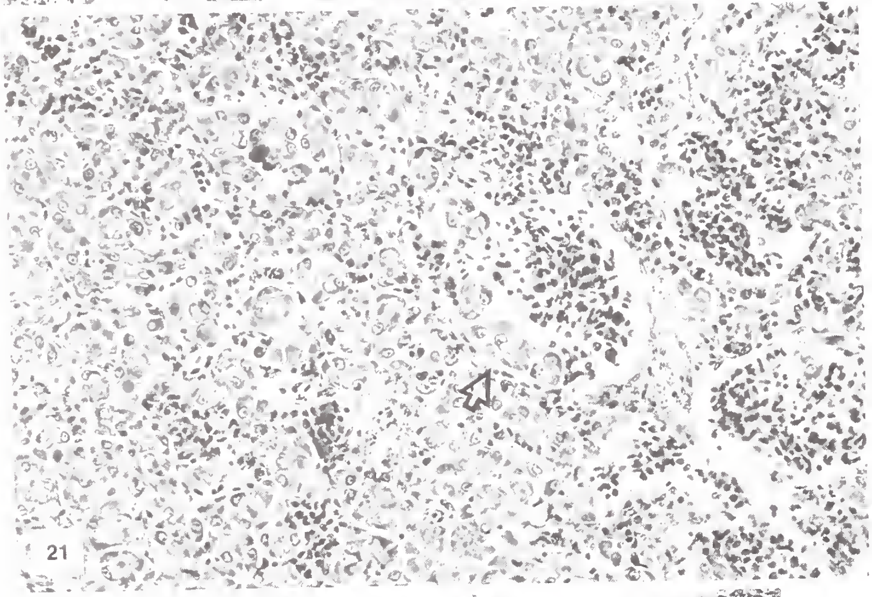

FIGURE 21.—Thyroid follicles apparently within blood vessel within mesenteric mass (presumably spleen). Treatment: 27° C and 0.04% *N*-OH-FAA. H & E. × 330

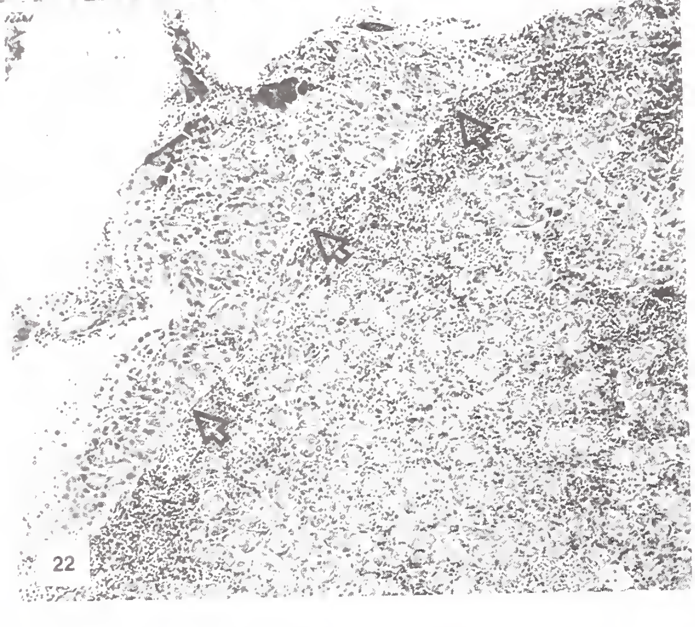

FIGURE 22.—Thyroid cells lying outside of capsule (*arrows*) of mesenteric mass containing thyroid tissue. Treatment: 27° C and 0.04% *N*-OH-FAA. H & E. × 170

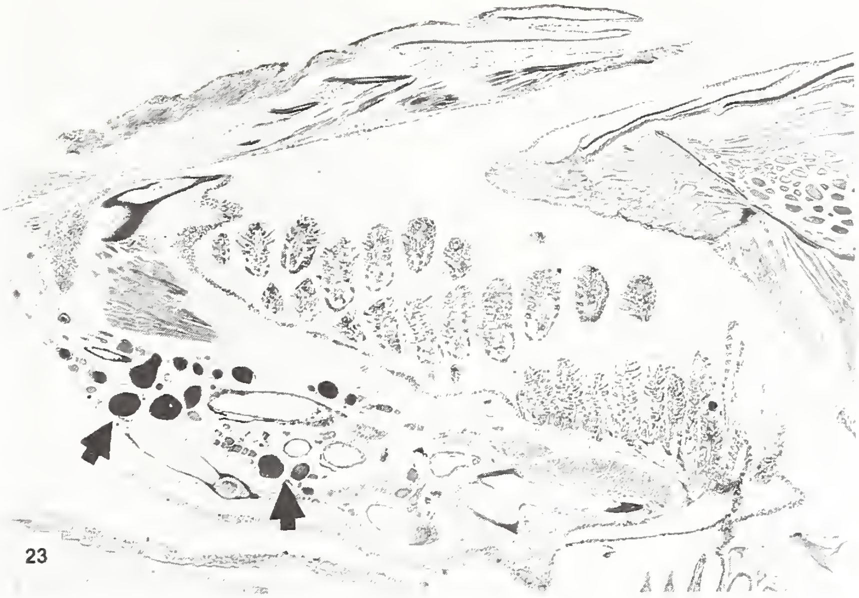

FIGURE 23.—Normal random distribution of thyroid follicles (prominently filled with colloid here) beneath gill chamber and running along course of ventral aorta. Treatment: 17° C and with no *N*-OH-FAA. H & E. × 54

test animals for carcinogens metabolically and physiologically handle a compound.

CONCLUSIONS

In this description of the unique features of the normal and proliferating teleost thyroid, I emphasize the caution that must be exercised in diagnosing a thyroid growth as neoplastic. Despite the seeming malignancy of these lesions in our experimental guppies, as evidenced by the marked local and distant growth, I diagnosed these lesions as hyperplasia, i.e., extreme benign proliferations of the pharyngeal and ectopic thyroid. Unfortunately, I could not determine the response of the guppies to iodine treatment before they were killed and thus cannot state unequivocally whether they would have responded to this widely recommended therapy.

In addressing the subject of this Symposium, I want to emphasize that thyroid hyperplasia, whether it is or can become neoplastic, remains a common and often environmentally related problem among numerous small fish species in captivity. Although the multiple functions of the fish thyroid have not been sufficiently clarified, we do know that triiodothyronine and thyroxine mediate the differentiation and growth of bone and probably influence metabolic and osmoregulatory functions in other tissues as well. Physiological alterations brought about by an abnormally functioning thyroid could change the responses of different tissues to a test compound and indirectly influence the outcome of a long-term bioassay. Thus it is important that one allow for adequate iodine in the water and diet before testing is initiated.

On the positive side, such a fascinating growth in which invasive behavior can sometimes be curtailed by treatment with iodine or other metals and from which some species can apparently "spontaneously" recover provides an excellent opportunity for one to explore and perhaps reexamine our definition of cancer. The apparent influence of environmental conditions on genetic background in leading to this proliferative lesion provides an excellent animal model for further exploration of the interaction between the two factors.

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DISCUSSION

J. Rice: Is it absolutely clear that these remote foci do constitute true ectopic foci, rather than, in some cases, at least, actual metastatic phenomena?

K. L. Hoover: Again, I would have to refer to Baker-Cohen's work. She followed this progressive increase of thyroid tissue that appeared to follow the vessels along the pathway, and, for a metastatic phenomenon, it really does not make sense when the flow of blood is taken into consideration. It would not appear in those sites if it was being carried through the bloodstream. As I said, she found it simply migrating along the surface.

Rice: So you would say that the criterion of invasion as used in mammalian tumor pathology would have to be rather extensively redefined?

Hoover: I do not know. I believe, in fact, that it might be necessary. It is an extremely interesting phenomenon: Gaylord originally described the vascular invasion by thyroid follicles within the pharyngeal area as well as an impressive regression of many of these growths following treatment with iodine.

J. Hendricks: Dr. Hoover, do you see many mitoses in these lesions?

Hoover: No, I have not.

F. Anders: I should comment that hybrids between *Xiphophorus maculata* and *X. montezumae* develop thyroid tumors spontaneously.

A. Woodhead: I have to raise this question, though I have talked with Dr. Hoover about this before. We treated the Amazon mollies with polycyclic hydrocarbons, and we got exactly the same effect in the spleen. It looks almost as if you have taken a tumor and stuck it down there, because the spleen is completely invaded, and now we have the whole stage through where you get half spleen and half thyroid. There are problems here about which I think I would like to ask questions. First of all, you are assuming it is ectopic. I have taken that as one of the hypotheses, that it is ectopic and the 7,12-dimethylbenz[*a*]anthracene has stimulated it. I must have been through hundreds of Amazon molly spleens (untreated), and I cannot find any ectopic thyroid. Now, if you look in the kidney and it is ectopic, you will pick up 1 follicle.

We have also done tracer experiments and cannot get any uptake of iodine at all in the untreated spleen. It is hard for one to think that ectopic tissue is normally there, unless it is there as single cells, and you just do not see it at all. The other thing is, Baker-Cohen did not look, as far as I know, at the guppies. She was quoting some German work, and when you go back to the original paper, the ones in which the author describes the thyroid in the spleen, i.e., 1 or 2 follicles were in fish that had been treated. They were not untreated animals. There is this whole question that, normally, I am not able to pick up any ectopic tissue in the spleen.

On the other hand, as you say, if you talk about migration, there is no way that ectopic tissue is going to turn up in the spleen, if it is going by a vascular route, with the exception that it goes through the kidney first. You have these massive spleens, yet you do not see anything in the kidney. The whole problem is most difficult to sort out.

Hoover: Yes. I think, though, that the ectopic tissue does not necessarily have to be producing colloid, which is, of course, the marker of the functioning thyroid. Perhaps, then, it would be extremely difficult to pick up such ectopic tissue.

Woodhead: Even with a radioactive label?

Hoover: Yes.

Woodhead: The other problem is that when you induce in the Amazon molly thyroid hyperplasia, or whatever you like to call it, by other means, you do not see anything in the spleen. As far as I am concerned, it is only true with polycyclic hydrocarbons.

B. Trump: Dr. Hoover, has anyone done any experiments in which one could mince up and actually inject thyroid tissue in iodine-deficient fish and study the patterns because the patterns of metastatic spread or normal tissue spread might well not reflect blood flow. We know that in the mammalian system there is the fertile soil concept. For example, although the blood flow through there is tremendous at a slow rate, the spleen is not normally a good site. That was one question. The other question is: Has any neoplastic-like development occurred in the A, B, or D cells like the C cell-type tumors? Or is that a component in the thyroid? Do fish have those A, B, or D-type cells in the thyroid?

Hoover: No, at least, not that I am aware of.

C. J. Dawe: The ultimobranchial body is the site of the C-type cells.

Hoover: Are there tumors in the ultimobranchial body?

Dawe: I do not know of any tumor in that organ.

K. Kator: Have you ever checked the thymus? In the medaka, we often see invasion of the thyroid into the thymus in normal fish.

Hoover: No, I have not. That is most interesting. The only “foreign” cells that I have seen in the thymus are the rodlet cells but never thyroid tissue.

J. Black: This is just a comment which I think would be worth sharing with the audience. Dr. Gaylord was the second director of Roswell Park Institute, which was the first institute devoted exclusively to the diagnosis and treatment of cancer.

As you are certainly well aware from having read Dr. Gaylord’s classic papers in the field of fish thyroid pathology, he was thoroughly convinced at the time that these lesions were, in fact, carcinomas. So he became so enthusiastic that he actually went out and acquired a farm south of the city of Buffalo that had an abundant supply of spring water with the intention of setting up a research study devoted to use of the thyroid hyperplasias as we now know they are, as a model of carcinoma of the thyroid. Of course, the discovery that these tumors regressed under the influence of iodine punched a hole in that whole idea. Even though the Roswell Park Institute had acquired this beautiful property with the springs, a few ponds were actually developed on the property at the time for growing trout, but they were never really used for trout until last year, when we installed a small trout hatchery on the property. So it has gone full circle, and now, at least, we finally are going to do some embryo exposures and raise some trout.

Hoover: I would just like to comment on that because he did address the fact that not only iodine but arsenic, apparently, cured these thyroid carcinomas. His point was that this further showed that these were true neoplasms because some studies in mice at the time were showing the tumors responded very well to certain elements in this chemical class. He was even more convinced as to their malignant nature.

Morphological Survey of Teleost Organs Important in Carcinogenesis With Attention to Fixation[1, 2]

David E. Hinton, Elizabeth R. Walker, Carlin A. Pinkstaff, *and* Edwin M. Zuchelkowski[3, 4]

ABSTRACT—Key features of the gross and microscopic anatomy of teleost liver, digestive tract, kidney, and skin were reviewed from the world literature. Illustrations of the above and new findings were obtained from light as well as scanning and transmission electron micrographs of tissues from 7 freshwater fish species: *Alosa sapidissima,* American shad; *Esox lucius,* northern pike; *Lepomis macrochirus,* bluegill sunfish; *Pimephales promelas,* fathead minnow; *Stizostedion vitreum,* walleye; *Oryzias latipes,* Japanese medaka; and *Ictalurus nebulosus,* brown bullhead catfish. Emphasis was placed on structural features that may influence interpretation of toxic and carcinogenic lesions. A discussion of fixation of small fish for light and electron microscopy and histochemistry is presented. — Natl Cancer Inst Monogr 65: 291–320, 1984.

Suitable animal models are needed if we are to assess changes in water quality with respect to potentially carcinogenic substances (*1*). Bioassay of fish may provide a reasonable means of gaining such information because tumors in feral fishes have been reported in virtually all organ systems (*2–4*). In addition, aquarium fishes (*5–11*) and the Mount Shasta strain of rainbow trout (*12*) have proved sensitive to certain carcinogens.

Our ability to detect anatomical alteration related to neoplasia in fishes depends on several important factors, some of which include: a thorough understanding of normal fish anatomy and histology; routinely adequate methods of fixation, processing, and staining to enhance detection of microscopic change; and inherent characteristics of individual fish species that make them suitable for analysis of specific types of compounds.

Our purpose is to review key features of the gross and microscopic anatomy of organ systems (liver, digestive tract, kidney) sensitive to laboratory carcinogen exposures or forming significant interfaces between the aquatic environment and the internal fish tissues (skin), and to review fixation methods commonly used for subsequent morphological assessment. In addition to a review of the literature, we will draw from unpublished material currently being assembled for an atlas of microscopic anatomy of 5 freshwater teleost species (*Alosa sapidissima,* American shad; *Esox lucius,* northern pike; *Lepomis macrochirus,* bluegill sunfish; *Pimephales promelas,* fathead minnow; and *Stizostedion vitreum,* walleye) and from ongoing studies with *Oryzias latipes*, Japanese medaka, and *Ictalurus nebulosus,* brown bullhead catfish.

The liver is by far the most common site for laboratory induction of primary tumors in various fish species (*3, 11*). Epitheliomas have been induced by direct application of benzo[*a*]pyrene and 3-methylcholanthrene to the skin (*13*). Only 3 compounds, dimethylnitrosamine, nitrosomorpholine, and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine produced tumors in sites other than liver, i.e., intestine (*7*) and kidney (*14, 15*).

LIVER MORPHOLOGY OF TELEOSTS

The arrangement of hepatocytes in most teleost livers has been described as tubulosinusoidal (*16*). In this pattern, plates or muralia are commonly 2 cells thick (*muralium duplex*) (*17*) and radiate out from a central vein (*18–20*). Abundant branching and anastomosing normally occur (*20, 21*), and, in appropriate orientation of sections, the pattern can appear as a continuous sheet of parenchymal cells (*8–10*). A typical field from the liver of the fathead minnow shows some features of the dual-plated muralium (fig. 1). In the tubulosinusoidal liver, the lumen of the tubule is the bile canaliculus (*16*), whereas the sinusoids are related to the basal aspects of hepatocytes across the space of Disse (fig. 2). When the plane of section lies perpendicular to the axis of the tubule (fig. 3), it is not uncommon for one to see small, oval nuclei of the bile preductular lining cells (*22*) which, by transmission electron microscopy, can be seen to share junctional complexes with hepatic parenchymal cells and to contribute microvilli to the bile canaliculus. Livers of goldfish (*23, 24*), zebra fish, mosquito fish (*25*), and the fathead minnow (this paper) contain so-called intracellular bile canaliculi. As is illus-

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ABBREVIATION: H & E = hematoxylin and eosin.

[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Supported in part by the Water Research Institute, West Virginia University, with funds allocated under the Water Resources Act of 1964 (PL 88-379), administered by the Office of Water Research and Technology, U.S. Department of the Interior, as part of Project A-037-WVA; by the Fish and Wildlife Service, Department of the Interior, contract No. USF & WS 14-16-0009-42; and by grant IN-127-A from the American Cancer Society.

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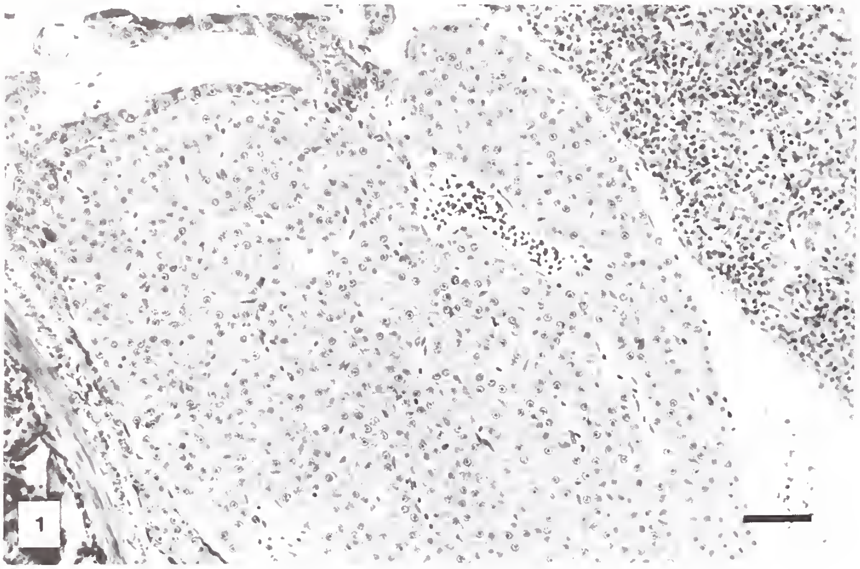


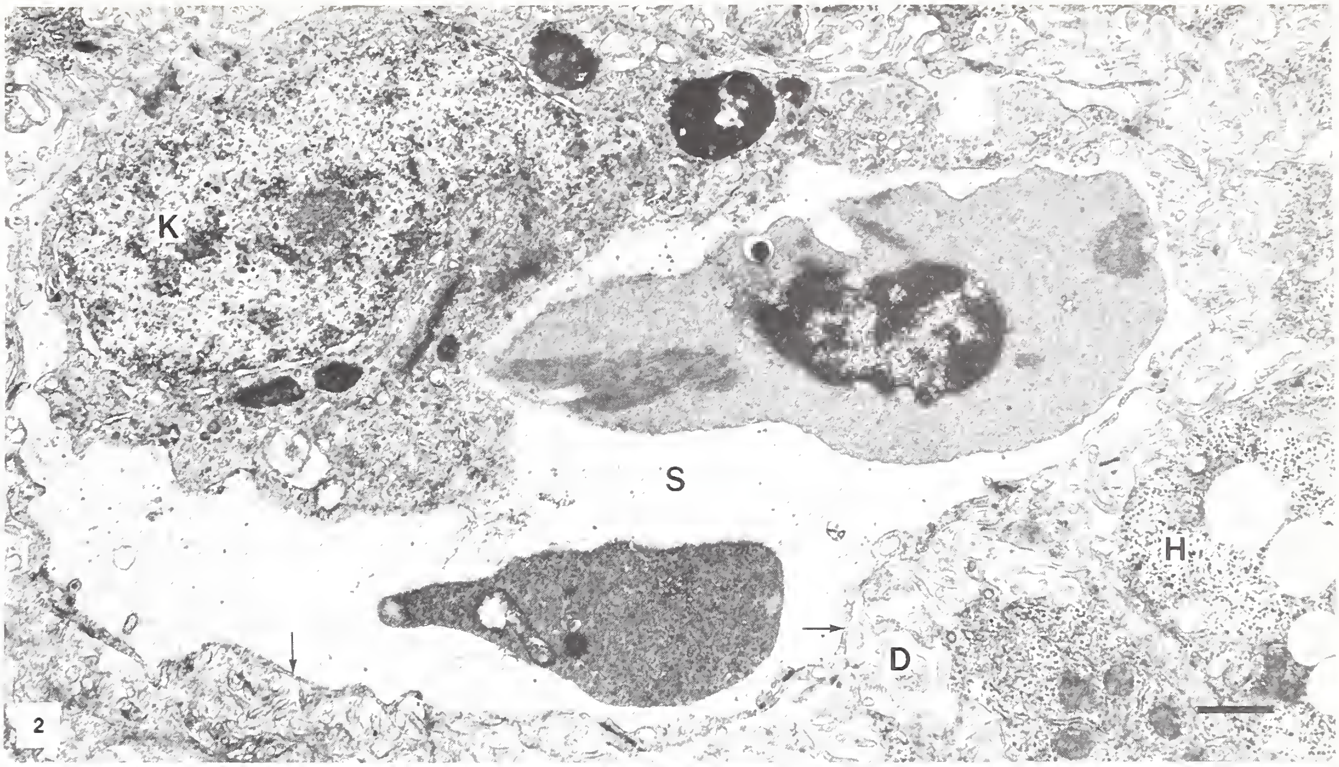
FIGURE 1.—Photomicrograph of paraffin section of fathead minnow liver showing the characteristic dual-plated muralium occurring in teleosts. Fixation in Bouin's solution. H & E. Bar= 10 μm. × 870

trated in figure 4, these are circular indentations of hepatocyte plasma membrane filled with microvilli-like projections from the same cell. By comparison, a typical bile canaliculus (fig. 5) from liver of *Lepomis macrochirus* shows microvilli from adjacent hepatocytes in the canalicular lumen. The hepatocytes share a junctional complex. David (*23*) interpreted his findings to mean that intracellular canaliculi discharged their contents into the interhepatocellular space. Such an interpretation can be obtained from figure 6. This section from *O. latipes* shows a pitlike depression in a hepatocyte. Microvilli extend into the depression, and the lumen is continuous with the intercellular space. Yamamoto (*24*), however, reported that intracellular bile canaliculi empty into terminal bile ducts, which was his designation for the bile preductules of Steiner and Carruthers (*22*). Weis (*25*) demonstrated connections between canaliculi with intracellular canaliculi draining into intercellular canaliculi.

Other fish species, such as *Ictalurus punctatus* (*19*) and *Salmo gairdneri* (*26*), although studied extensively, apparently have no intracellular canaliculi and in this regard more closely resemble the mammalian liver (*23, 27, 28*). Collections of bile preductular cells forming an intralobular biliary ductule (duct of Hering) are seen in figure 3. From here the drainage occurs into interlobular ducts, intrahepatic and extrahepatic ducts, and finally into the intestinal lumen (*23*). In our analysis of various fish species, we have been impresssed by the abundance of bile preductular epithelial cells in the teleost liver lobule. Perhaps this close association explains in part the common finding of tumors with both hepatocellular and biliary epithelial constituents (*5-9*).

The perisinusoidal space of Disse, located between the basal aspects (tubulosinusoidal model of liver architecture) of the hepatic parenchymal cells and the sinusoidal endothelium, contains numerous finger-like projections from hepatocytes (fig. 2). Teleost perisinusoidal cells include the fat-storing cells of Ito (*28*). These are found in nearly all species [Ito, Watanabe, Takahashi as cited in (*29*)] and are particularly abundant in arctic fishes (*30*). When cyprinid fish are exposed to vitamin A, so-called empty fat-storing cells undergo changes resulting in dilation of cisternae of granular endoplasmic reticulum, increased numbers of microfilaments and microtubules in cell processes, and appearance of these microfilaments and microtubules in cell bodies (*29, 31*). If the exposure to vitamin A is of sufficient duration (*29*), fat droplets will appear in previously empty fat-storing cells. Based on the location and ultrastructural features, Tanuma and Ito (*29*) regarded a new type of perisinusoidal cell, described in Nopanitaya et al. (*32*), as an empty fat-storing cell. Inasmuch as a tonofilament-rich, bile preductular cell can be among the cells bordering the canaliculus (*33*), precise identification of cell types, other than hepatocytes, depends on an understanding of their orientation to the neighboring hepatocytes (*29*). The perisinusoidal cells and their relationship to hepatic parenchymal and endothelial cells are well illustrated and described in a study of *Carassius carassius* (*29*). Recently, Ferri (*34*) described ultrastructural features of another cell type (pit cells) in the liver of the teleost, *Pimelodus maculatus.* These cells, with characteristic electron dense granules, were seen in liver capsules, sinusoidal lumina, near biliary ductules, between hepatocytes, and in subendothelial locations. Because these cells showed no evidence of endocytotic activity after India ink injection and because their granules resembled those of enteroendocrine cells in mammals, birds, and fishes, an endocrine function has been proposed.

Kupffer cells, apparently few in number, are found in some fish species (*19, 31*). Evidence for their phagocytic function was the presence of heterophagic vacuoles containing recognizable organelles (*19*), and morphologic evidence suggesting phagocytosis of heterogeneous erythrocytes (*31*). Papers by Hacking et al. (*26*) and Fujita and associates (*31*) contain reviews of this literature. In the liver of *Alosa sapidissima,* we found Kupffer cells (fig. 2) similar



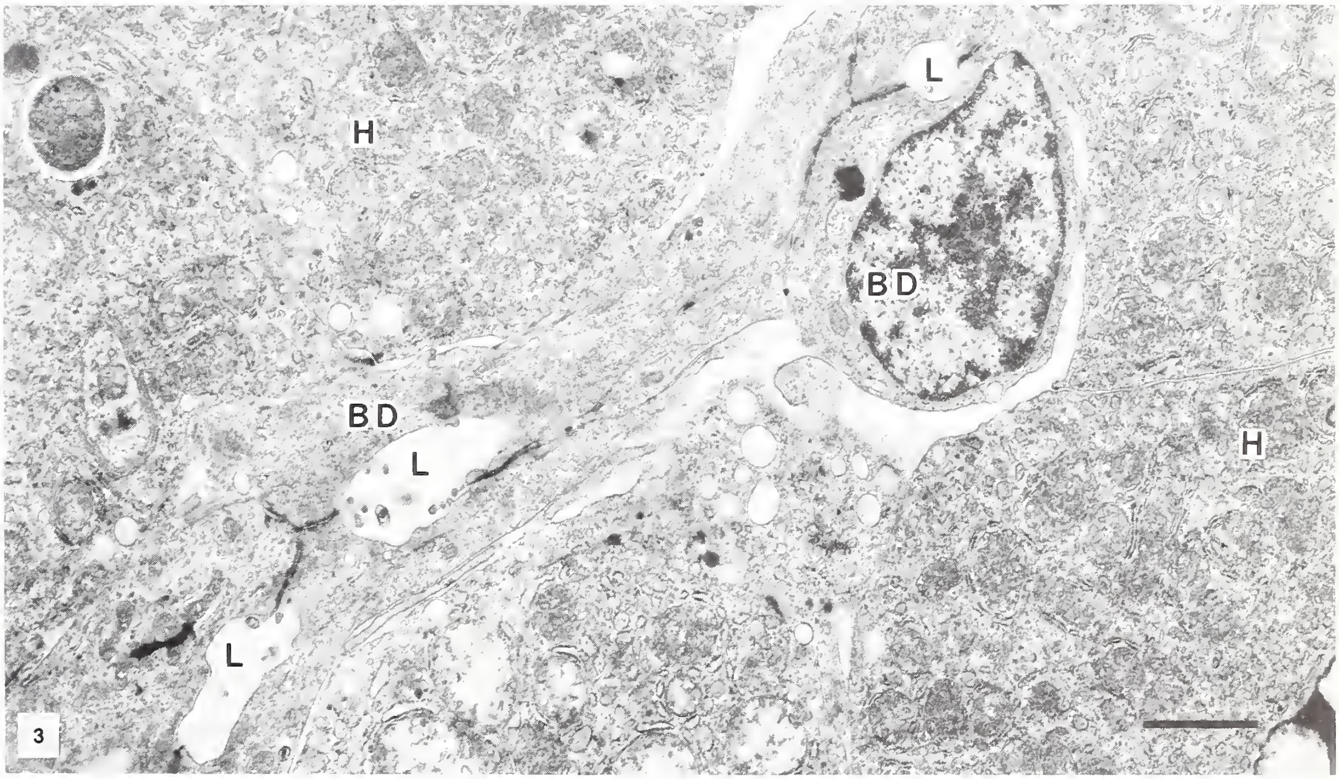


FIGURE 2.—Sinusoidal region from liver of American shad shows a single Kupffer cell (K), with cytoplasmic electron dense bodies. Attenuated endothelial cells (*arrows*) separate sinusoidal lumen (S) from perisinusoidal space of Disse (D) in which finger-like projections of a hepatocyte (H) are seen. Immersion fixation in 0.1 *M* phosphate buffered 3% glutaraldehyde followed by postfixation in 2% osmium tetroxide. Uranyl acetate and lead citrate. Bar = 1 μm. × 10,000

FIGURE 3.—Bile preductule in liver of bluegill sunfish is surrounded by hepatocytes (H). Bile preductular lining cells (BD) surround lumen (L) in this longitudinal section. Fixation is as in figure 2 legend. Uranyl acetate and lead citrate. Bar = 1 μm. × 15,000

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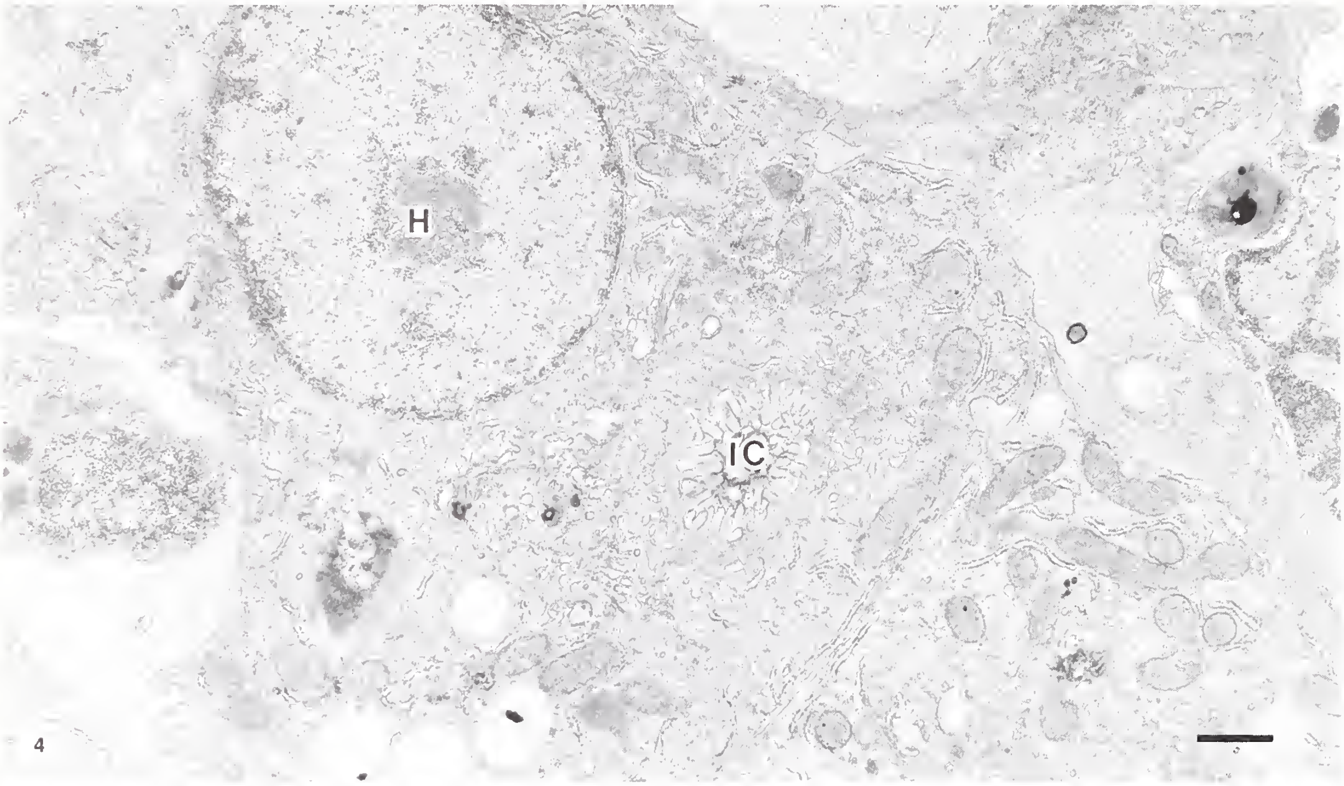

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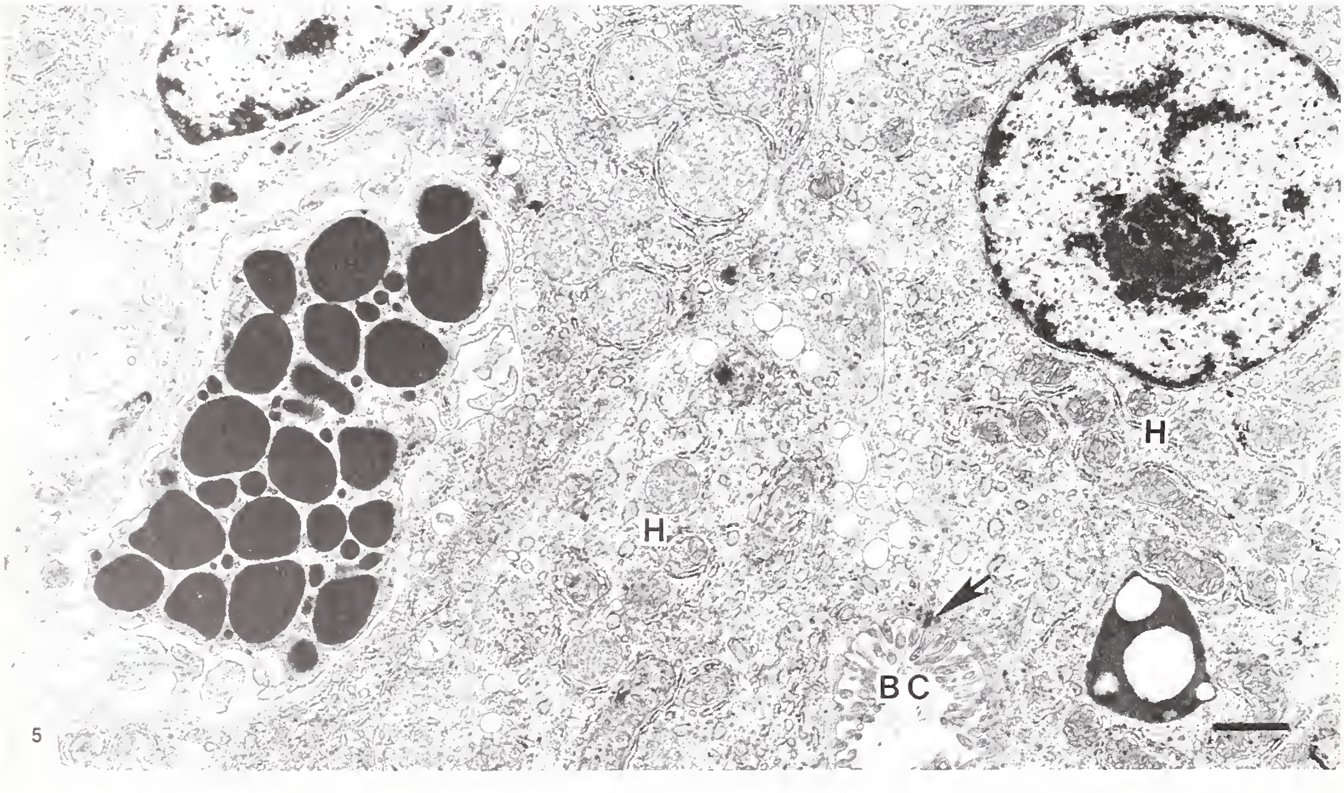


FIGURE 4.—Intracellular canaliculus (IC) in a hepatocyte (H) of fathead minnow. Fixation as in figure 2 legend. Uranyl acetate and lead citrate. Bar=1 μm. × 10,000

FIGURE 5.—Intercellular bile canaliculus (BC) between hepatocytes (H) of liver from bluegill sunfish. *Arrow* points to junctional complex between hepatocytes. Fixation as in figure 2 legend. Uranyl acetate and lead citrate. Bar=1 μm. × 10,000

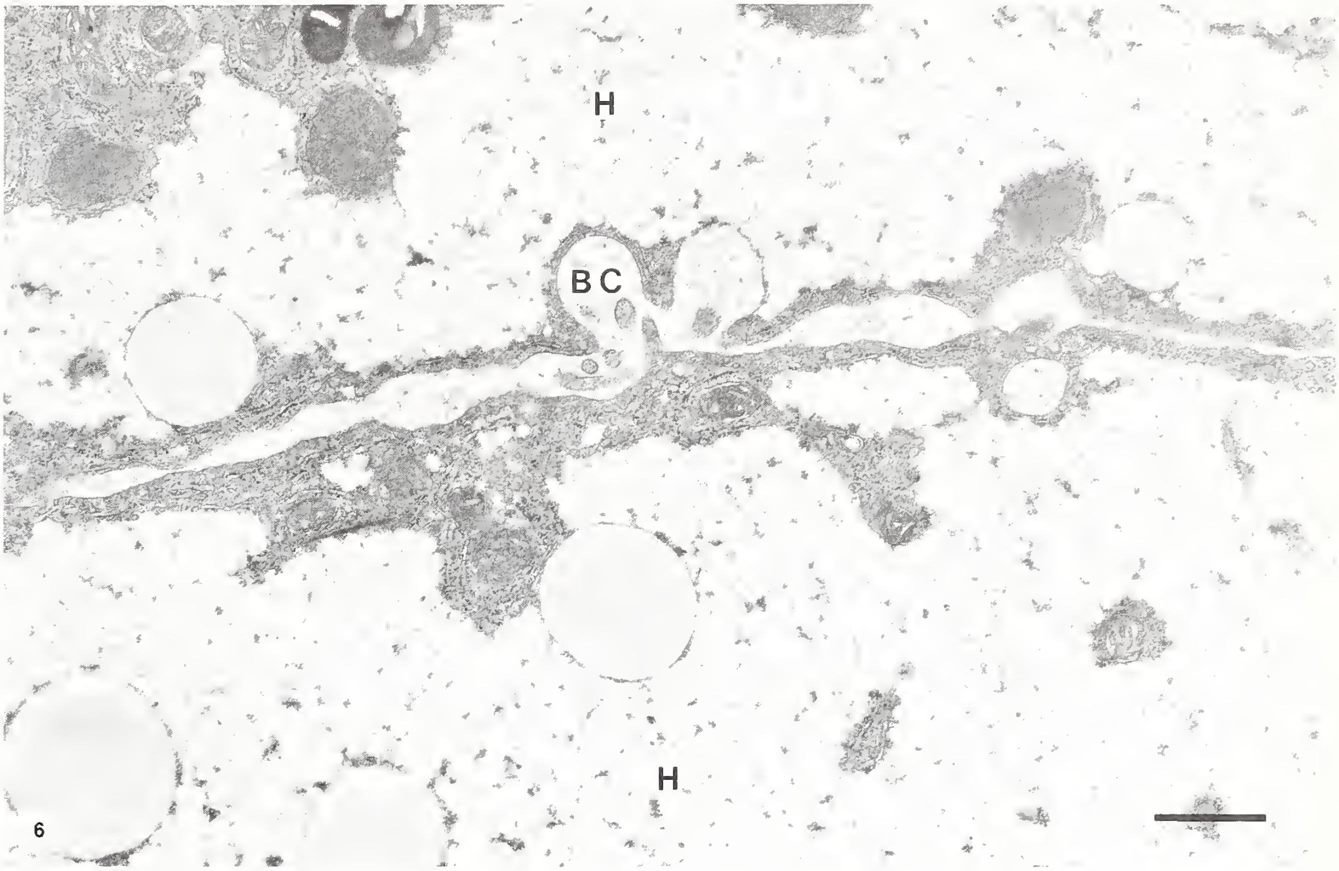


FIGURE 6.—Intercellular bile canaliculus (BC) between hepatocytes (H) of liver from Japanese medaka. Fixation as in figure 2 legend. Uranyl acetate and lead citrate. Bar = 1 μm. × 15,000

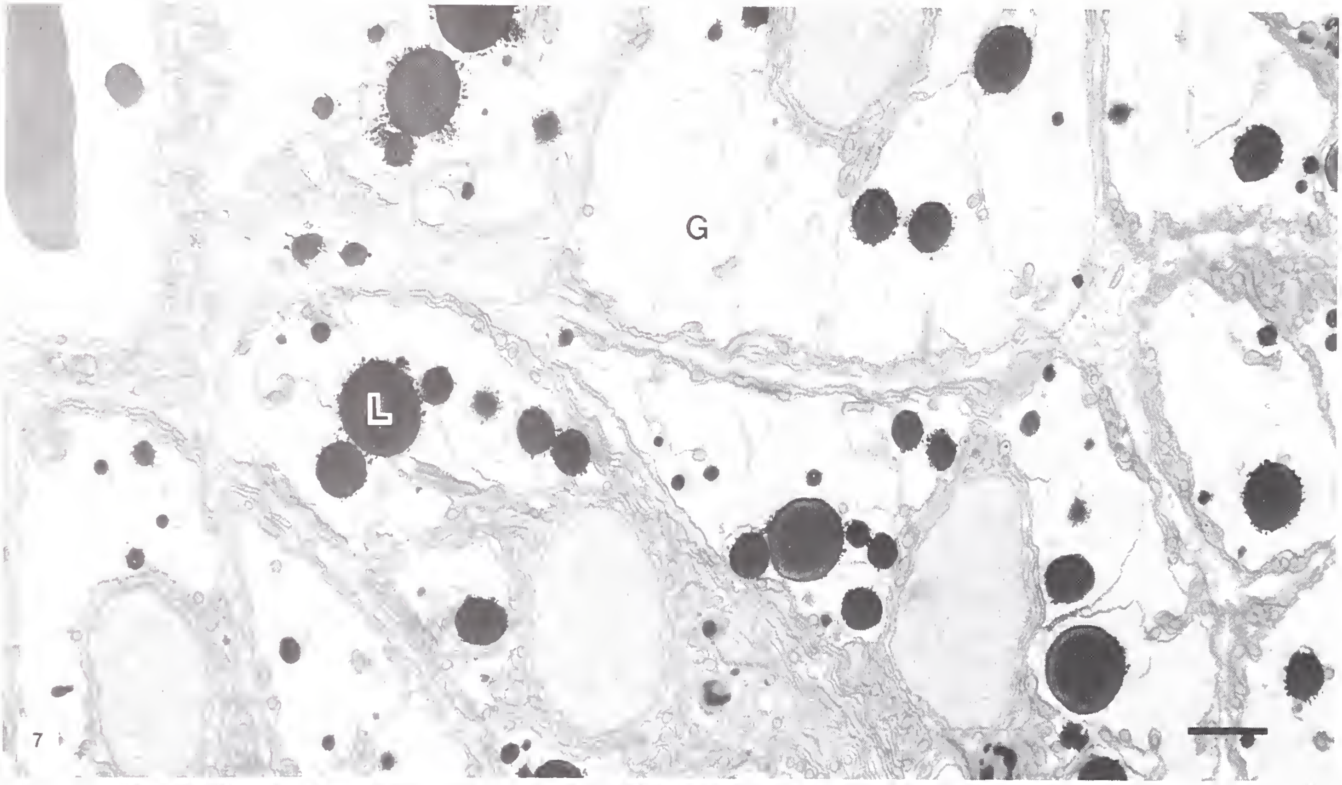
in appearance to those of an earlier report in *I. punctatus* (*19*).

Histologically, lobules in teleost liver are indistinct (*20*) or absent (*8, 24, 35*). Although only limited enzyme histochemical data are available (*18, 33, 35*), a concentration of reaction product within regions of hepatic parenchyma (i.e., near the central vein or portal vein) is an infrequent finding. Authors of 1 study localized reaction products for 11 liver enzymes by way of histochemical procedures in 8 species of teleosts (*33*). In 1 species, *Dascyllus trimaculatus*, the reaction product for β-glucuronidase was concentrated around the central veins. In all species, enzyme reactions showed no zones of concentration.

Electron microscopic observations on teleost hepatocytes have been reported on various species (*8, 18, 19, 25, 26, 30, 33*). Ultrastructural similarities have emerged that appear to support the following general description. Teleost hepatocytes commonly contain a rounded nucleus (figs. 4, 5, 7, 8) with a single nucleolus and large regions of euchromatin. When primary aldehyde fixation is used, heterochromatin is found at the periphery of the nucleus. Cytoplasmic ultrastructure includes a ring or "perinuclear cuff" (*20*) of endoplasmic reticulum and mitochondria. Extensions of endoplasmic reticulum, mitochondria, and occasional dense bodies or presumptive lysosomes extend from this central location toward the cell periphery (fig. 7). Variable amounts of glycogen are found (figs. 7, 8) that may reflect species variation (*33*), nutritional status, and diet formulation (*18*). Glycogen may occupy large areas within the cytoplasm (fig. 7) or be restricted to smaller regions (fig. 8); in the smaller regions, the relative amount of endoplasmic reticulum- and mitochondria-containing zones of the cell appear increased (fig. 8). Agranular endoplasmic reticulum is found at the periphery of granular endoplasmic reticulum and in glycogen-containing zones (*18, 19, 36, 37*). Upon appropriate exposure to specific xenobiotics, the endoplasmic reticulum may be stimulated to proliferate, and, in these instances, membranous whorls (*18, 26, 37, 38*) may be seen. Lipid vacuoles are found within the same areas of the cytoplasm which contain glycogen (fig. 7). The amount of lipid varies between species (*33*) and apparently with the season in some species (*39*).

Acini of exocrine pancreas are found to a variable extent in walls and periadventitia of portal veins (*19*). In the liver (fig. 9), the dual association of these organs has led to the term "hepatopancreas." Interestingly, we have found acini in walls of veins in the spleen of *P. promelas* (fig. 10). It is possible that exocrine pancreatic acini can be found in association with any of the tributaries of the hepatic portal vein.

In typical H & E-stained liver sections (fig. 11), cyto-



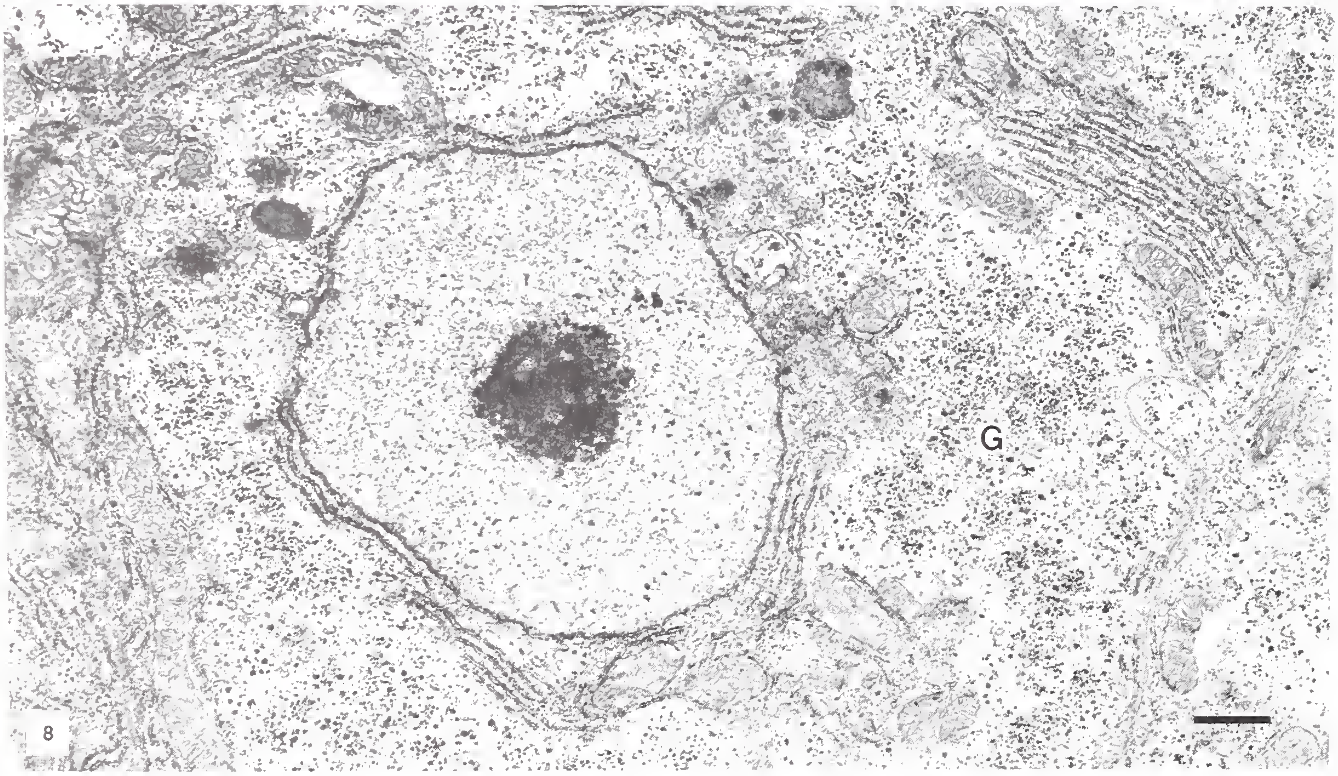


FIGURE 7.—Section through hepatocytes from liver of northern pike. Each cell contains several lipid droplets (L) and large areas of cytoplasm are filled with glycogen (G). Fixation as in figure 2 legend. Uranyl acetate and lead citrate. Bar = 2 μm. × 5,200

FIGURE 8.—Hepatocyte from liver of walleye shows a perinuclear cuff of endoplasmic reticulum and mitochondria. Abundant glycogen (G) is also seen. Fixation as in figure 2 legend. Uranyl acetate and lead citrate. Bar = 1 μm. × 10,000

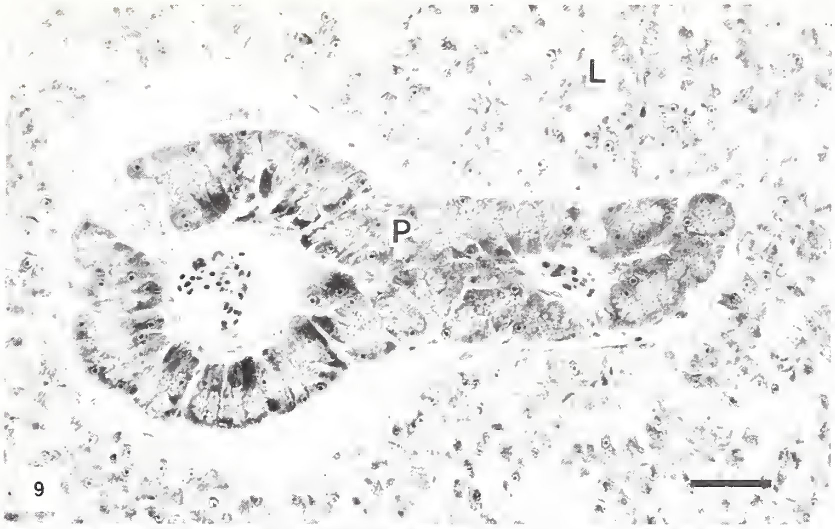


FIGURE 9.—Photomicrograph of paraffin-embedded walleye liver (L) showing exocrine pancreatic acini (P) in wall of portal vein. Dietrich's fixative. H & E. Bar = 10 μm. × 1,070

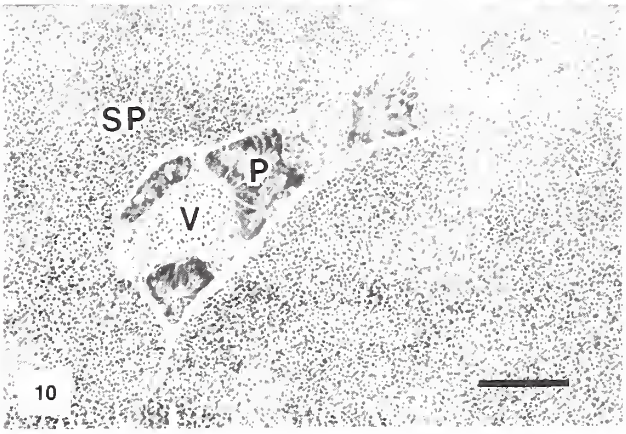


FIGURE 10.—Acini of exocrine pancreas (P) in close association with a vein (V) are shown in this photomicrograph of paraffin-embedded fathead minnow spleen (SP). Fixation in Bouin's fluid. H & E. Bar = 100 μm. × 120

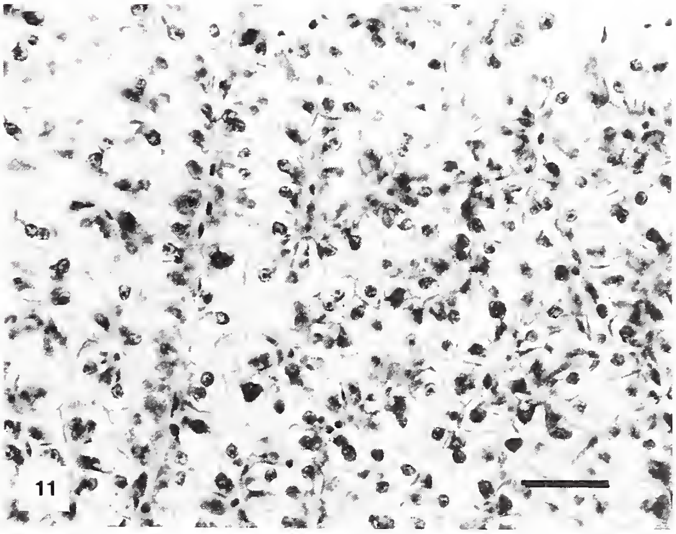


FIGURE 11.—Photomicrograph of paraffin section of walleye liver. Clear unstained zones in hepatocytes correspond to areas of glycogen accumulation seen in electron micrograph (fig. 8). Fixation in Bouin's solution. H & E. Bar = 30 μm. × 380

plasmic regions related to glycogen accumulation appear as large unstained areas. Variable amounts of basophilic material are found immediately around the nucleus, as small clumps within the cytoplasm, and occasionally concentrated at the cell periphery. One can usually determine individual cell boundaries of hepatocytes at the light microscopic level.

Any survey of teleost hepatic morphology would be incomplete without mention made of the collections of macrophages termed "melanomacrophage centers". These have been reviewed in detail with relation to disease in teleosts (*40*). Their presence was noted during early degenerative phases of carcinogen exposure in small aquarium fishes (*5*).

DIGESTIVE TRACT MORPHOLOGY OF TELEOSTS

In early descriptions of the gross and light microscopic anatomy of the digestive tract in various teleost species (*41–50*), this organ system is divided into: buccal cavity, pharyngeal cavity (associated with branchial arches), esophagus, stomach (or intestinal bulb in agastric teleosts), and the intestine. The dissected walleye digestive tract (figs.

12 15) illustrates general gross anatomical features of teleost digestive tracts. A modified tongue, in the floor of the buccal cavity, is connected with the branchial arches in the ventral midline. Caudal to the branchial arches, the cavity of the digestive tract constricts and the mucosa is thrown into longitudinal folds at the tubelike esophagus which extends from the pharynx to its junction with the corpus of the stomach (*51, 52*). In figure 13, the cul-de-sac (gastric cecum) of this cecal-type stomach (*52*) is shown, and portions of 2 intestinal ceca (*53, 54*) can be seen protruding from behind the stomach. In figure 14, a ventral view of this digestive tract reveals the point of attachment of the intestinal ceca. From its beginning, immediately distal to the pylorus of the stomach, the intestine proceeds caudally for a variable distance. After the first bend, the tube then proceeds rostrally to a second bend, after which the course of the intestine is a straight line projecting caudally to the anus. Regions similar to the mammalian small intestine, large intestine, and rectum cannot be identified in undissected teleostean intestines (*55*). Furthermore, regional differences in dissected specimens are inconstant and apparently are related to species. Reifel and Travill (*55*) studied a series of 10 teleostean species, selected on the basis of diverse feeding habits, and, after longitudinal sectioning, identified a thicker walled rectum and an intestinorectal valve in only 4 species. A slightly magnified view of the dissected liver, biliary system, and gall bladder is shown in figure 15. The association of common bile duct with cystic duct and hepatic ducts, and the entry of the common bile duct into the initial portion of the intestine are also visible.

Light microscopic examination of the buccal cavity reveals a stratified cuboidal epithelium containing mucous and nonmucous epithelial cells (*41, 43–46, 50*). The epithelium of the esophagus is stratified cuboidal with mucous cells (*41–48, 50, 56*). Routine paraffin sections of the northern pike esophagus (fig. 16) reveal a mucosa which is thrown into longitudinal folds and has abundant skeletal muscle in the muscularis. By high-resolution light microscopy, both mucous and nonmucous cells can be seen in the stratified epithelium of the esophagus (fig. 17). Mucus-containing cells lie at the surface and deep within the stratified epithelium. Only a few ultrastructural studies have been done on the esophageal region in teleosts (*57–59*). The ultrastructure of esophageal mucous cells is shown in figure 18. Some of these cells are large and resemble mucus-containing cells present throughout portions of the digestive tract. Occasionally, a rounded cell with what may be a single secretory granule occurs (fig. 18); these have been referred to as "einkornzellen" by some German workers (*59*). Lining cells in the esophageal mucosa show surface specializations called microridges (fig. 18) which are shown particularly well by scanning electron microscopy (fig. 19). In teleosts, this microridge pattern (fig. 19) is thought to hold secretions on the surface of mucus-secreting epithelia, such as the esophagus, skin, and portions of gill (*60*). With scanning electron microscopy, an abrupt change from the microridge pattern on esophageal epithelium to the small bleblike structures characteristic of gastric epithelium occurs (fig. 19). However, standard histological specimens reveal a gradual transition in both muscularis (skeletal-esophagus; smooth-stomach) and mucosal epithelium (stratified cuboidal-esophagus; simple columnar with glands-stomach) at the esophageal–stomach junction (fig. 20).

Impressed by the length of this region of transition in *Calamoichthys calabricus*, Purser (*61*) introduced the term "esogaster," to signify the composite nature, partly esophageal and partly gastric, on this region which has received recent attention in a comparative study of 8 teleost species (*52*). Surface cells of the stomach, arranged as simple columnar epithelium, contain dark-staining material (mucus) in their apical regions. Mucosa of the rectilinear corpus of the stomach in a northern pike is thrown into folds when the gastric lumen is empty (fig. 21) or is smooth when the lumen is distended (fig. 22). Gastric glands (fig. 22), found within the mucosa in this region, are not seen in the esogaster (fig. 20). Since 1877, teleost gastric glands have been known to contain only one cell type (*45, 49, 52, 62*). This cell type, the "parieto-chief" cell, has structural features of both the mammalian parietal and the chief cells. Parietal cells containing prominent intracellular canaliculi are known to secrete acid, and chief cells containing zymogen granules synthesize the digestive enzyme, pepsin. The parieto-chief cell from a teleost gastric gland (fig. 23) contains both abundant intracellular canaliculi and zymogen granules.

Microscopically, segmentation of the intestine into 3 distinct zones has been reported in gastric (*54, 63, 64*) and agastric teleosts (*63, 65–71*). The first of these, a lipid absorptive zone, includes the intestinal bulb in cyprinids (*71*) and the intestinal ceca in gastric teleosts (*54*) and occupies approximately 65–75% of the intestinal length. The second or middle segment occupies 25–35% of the intestinal length (*72*). Here absorptive cells take up protein and other macromolecules by pinocytosis (*65*). The third segment is considered, based mainly on ultrastructural evidence, to be a water and ion absorptive region (*72*).

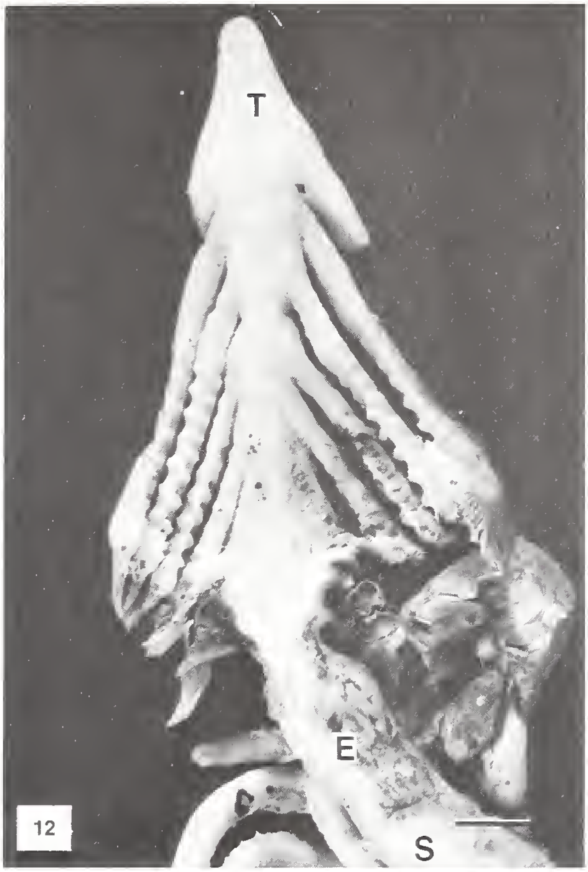
→

FIGURE 12.—Photograph of portion of dissected walleye digestive tract from a mature male of approximately 35 cm body length. A modified tongue (T) is connected to branchial arches in pharynx. The esophagus (E) extends from pharynx to junction with corpus of stomach (S). Bar = 10 mm.

FIGURE 13.—Photograph of region of walleye digestive tract adjacent to field in figure 12. Three intestinal ceca (C) protrude from behind the cecal-type stomach (S). Intestine (I) extends distally toward bottom of field. Bar = 6.7 mm.

FIGURE 14.—Dissected walleye digestive tract (ventral view). Relationship of four intestinal ceca (C) to intestine (I) and stomach (S) is shown. Bar = 7.1 mm.

FIGURE 15.—Photograph of dissected walleye liver (L), extrahepatic biliary tract, and intestine (I). The gall bladder (GB) is connected to the right hepatic duct (RHD) by a large cystic duct (CD). The common bile duct (CBD), formed by the junction of the right with the left hepatic duct (LHD), enters the intestinal wall near site of attachment of intestinal ceca. Bar = 10 mm.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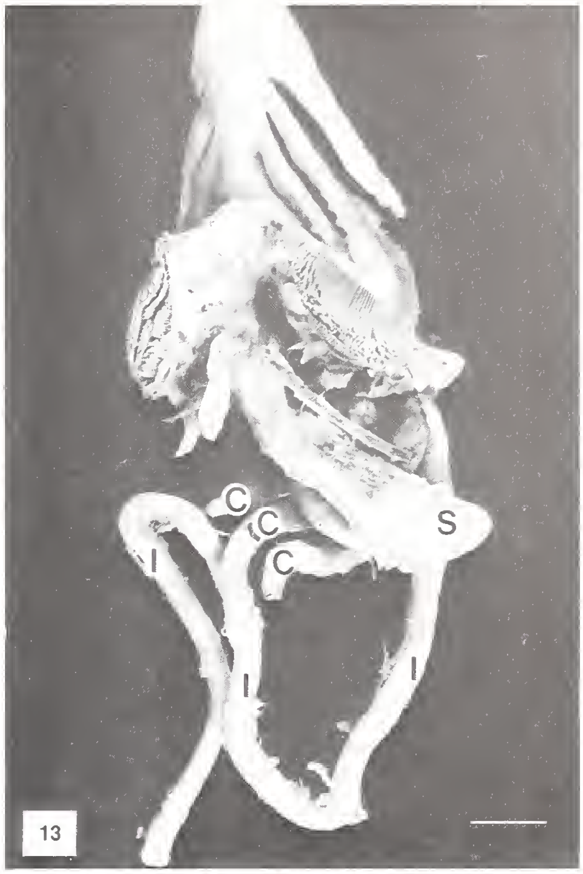
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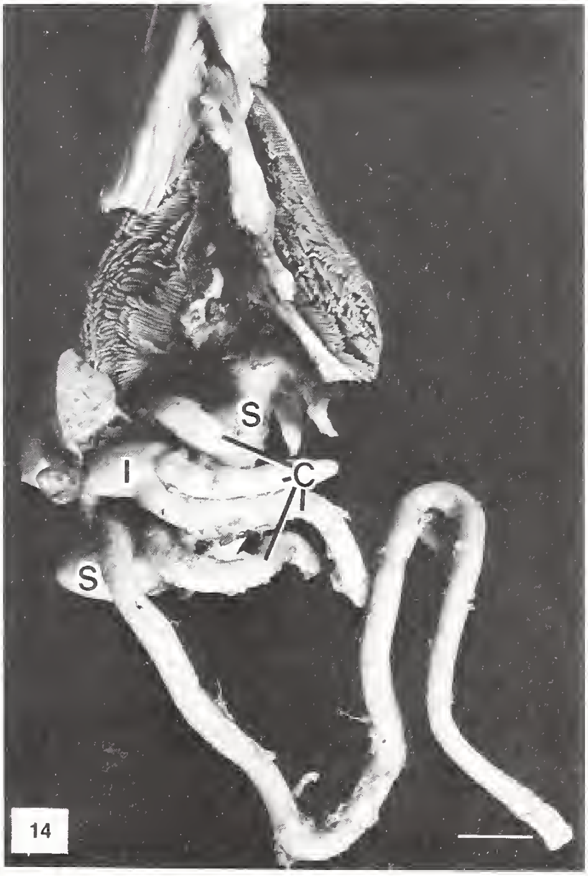
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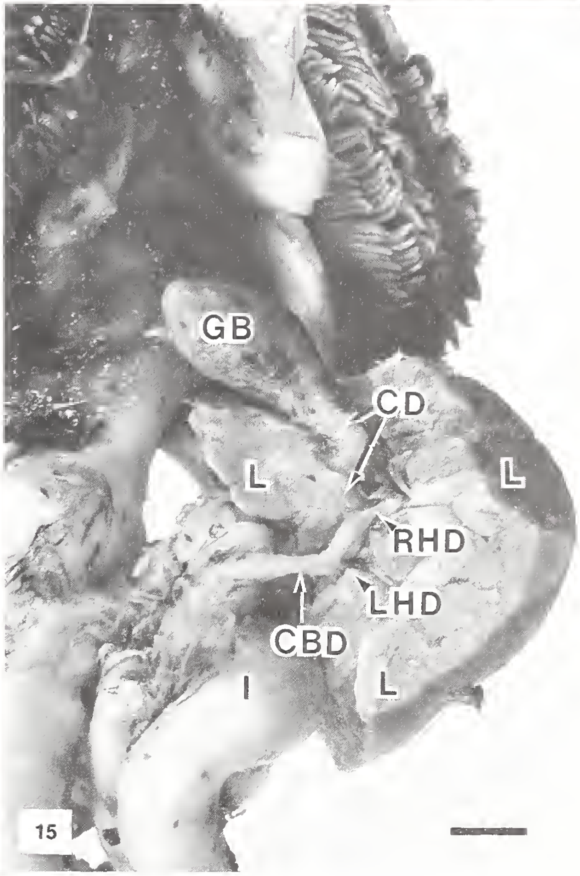
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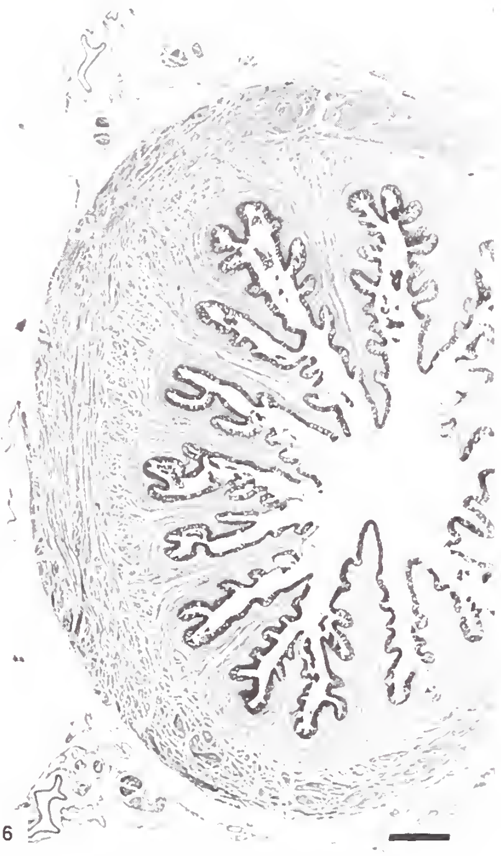

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FIGURE 16.—Photomicrograph of paraffin section through northern pike esophagus. Fixation in Bouin's fluid. H & E. Bar= 100 μm. × 80

Unfortunately, only 1 report links grossly observable intestinal features with the microscopic segmentation. Noaillac-Depeyre and Gas (*54*) observed a zigzag pattern in the mucosal folds of the first portion of the intestine in *Perca fluviatilis*. Mucosal folds overlying the middle segment were described as longitudinal, whereas those of the distal-most segment were reticular in pattern. All other accounts indicate no gross intestinal features indicative of the limits of the microscopic segments.

A convincing microscopic demonstration of this segmentation in teleosts can be accomplished by ingestion or intubation of a high fat diet and subsequent observation of cytologic (*64-66*) and biochemical changes (*66*) in the lipid absorptive zone. Figure 24 shows the ultrastructure of lipid absorptive cells from the intestinal bulb of the fathead minnow, and figure 25 depicts similar cells in the intestine of a gastric teleost. In both, the terminal web is of low electron density and agranular endoplasmic reticulum is sparse. Mitochondria, granular endoplasmic reticulum, lipid particles, and large lipid droplets are present. Particles of lipid less than 100 nm, similar to chylomicrons are apparently the transport form (*54, 64, 70, 71*). Larger droplets (1-5 μm) may be destined for intracellular storage (*54, 70*).

A protein absorptive cell from the walleye intestine is shown in figure 26. Abundant pinocytotic vesicles fill the terminal web region at the bases of the microvilli. The apical cytoplasm shows a dense tubulovesicular network which is part of the agranular endoplasmic reticulum. Fairly large cytoplasmic inclusions with clear or electron lucent portions extend from agranular endoplasmic reticulum toward basal aspects of cells and are associated with numerous lysosomes. Similar findings regarding this cell type have been reported in other teleostean species (*54, 63, 65-68, 71*). In the most distal portion of the intestine, lining epithelial cells have fewer and shorter microvilli and fewer pinocytotic vesicles. Also, more goblet cells are present (*67, 69*).

Enteroendocrine cells are found throughout the teleost digestive tract (*55, 72-76*). In the cyprinid teleost, *Barbus conchonius*, 4 types of enteroendocrine cells have been described (*72, 73*). The classification of these cells is based on size of their secretory granules and whether they extend to the lumen (open) or are located close to the basement membrane with no apparent luminal extension (closed). The open nature of some enteroendocrine cells is particularly well illustrated when histochemical (*72*) or immuno-

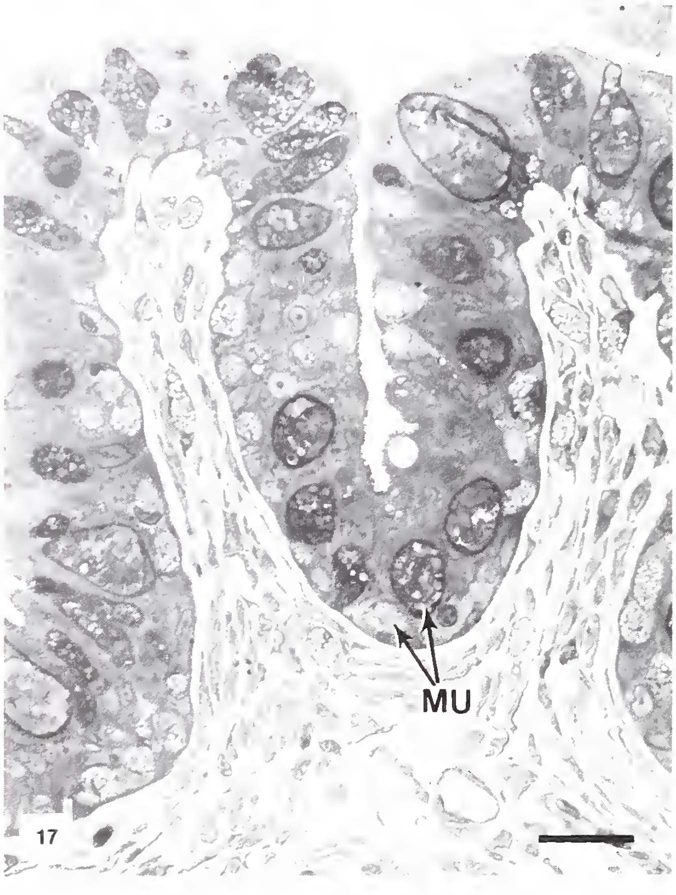

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FIGURE 17.—Photomicrograph of section through Epon-embedded northern pike esophageal mucosa. Mucous cells (MU) are seen at the surface and deep in the epithelium. Fixation as in figure 2 legend. Toluidine blue. Bar= 10 μm. × 1,260

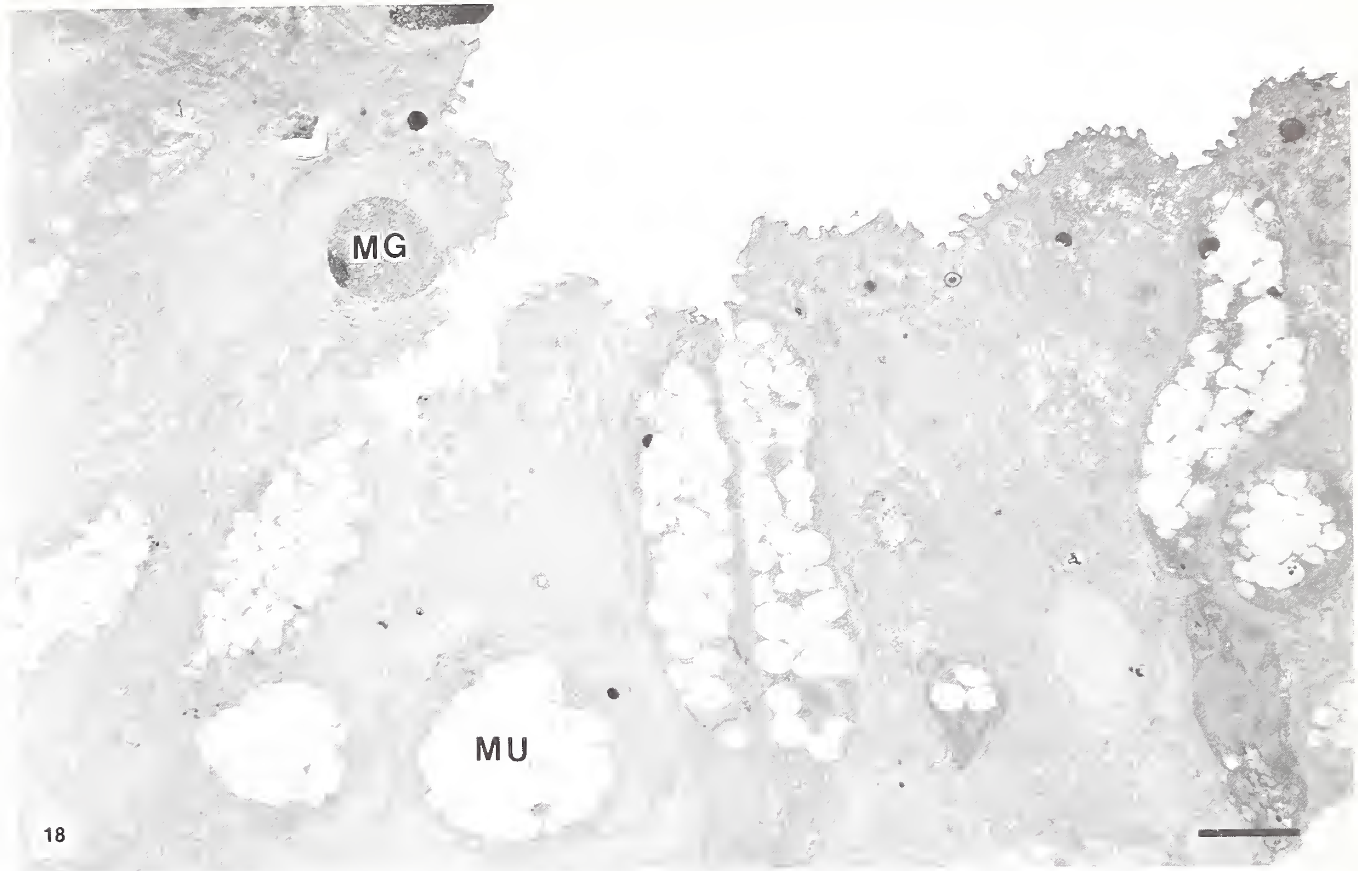

FIGURE 18.—Transmission electron micrograph of section through Epon-embedded northern pike esophagus (lining epithelium). A monogranular cell (MG) with a single electron dense (secretory?) granule can be seen. In addition, other mucus-containing cells (MU) are seen. Fixation as in figure 2 legend. Uranyl acetate and lead citrate. Bar=5 μm. × 2,600

cytochemical (*77*) methods are used. The number of enteroendocrine cells is said to decrease in the caudal direction, with few in the protein absorptive region of the intestine. In walleye stomach, we have seen enteroendocrine cells between basal aspects of parieto-chief cells and the lamina propria (fig. 27). In the intestine of the fathead minnow and the northern pike, these cells lie in the mucosal epithelium.

Regions within specific divisions of the digestive system are not necessarily uniform. Recent carbohydrate histochemical studies of mucosubstances in stomach (*52*), intestine (*55*), and esophagus (*56*), reveal that the secretory products within even highly restricted areas of the gut may differ. Reifel and Travill studied the gross anatomy and mucosubstance histochemistry of the digestive system in several species of fish (*51, 52, 55, 56*). Tables 1–4 present our preliminary findings on mucosubstance histochemistry of the American shad digestive system. Methods used for demonstration of mucosubstances in our study are described in detail by Spicer et al. (*78*), and a brief theoretical discussion of mucosubstance histochemistry is given by Lev (*79*). From this histochemical data, it can be seen that 3 regions exist in the esophagus and that the floor and roof of the mouth differ greatly. In addition, the corpus of the stomach and the pyloric stomach differ in the types of mucosubstances present.

MORPHOLOGY OF TELEOST SKIN

Analysis of normal morphology of teleost skin is important in considerations of laboratory-induced carcinogenesis and in analyses of tumors found in feral populations of fishes. Epidermal neoplasms have been reported in a variety of teleost species from both clean and heavily polluted waters (*80, 81*). Although some of these neoplasms have been classified as malignant, most teleost epidermal tumors exhibit a benign papillomatous structure (*80–84*).

To recognize neoplastic teleost skin, one must have a thorough understanding of normal epidermal structure at the light and electron microscopic level. Bullock and Roberts (*85*) presented the basic divisions of teleost skin in a review. From the outermost portion inward, the layers include: cuticle, epidermis, basement membrane, dermis, and hypodermis. The cuticle has been described as a delicate collection of dead and dying cells, cellular components, and secreted mucus. Under normal conditions, most of the glycoprotein component of the cuticle is believed to be secreted by surface epidermal cells (*86*). However, under conditions of stress, goblet cell discharge may be sufficient to dislodge the cuticle, replacing it with a glycoprotein secretion (*86*).

Early literature concerned with light microscopic obser-

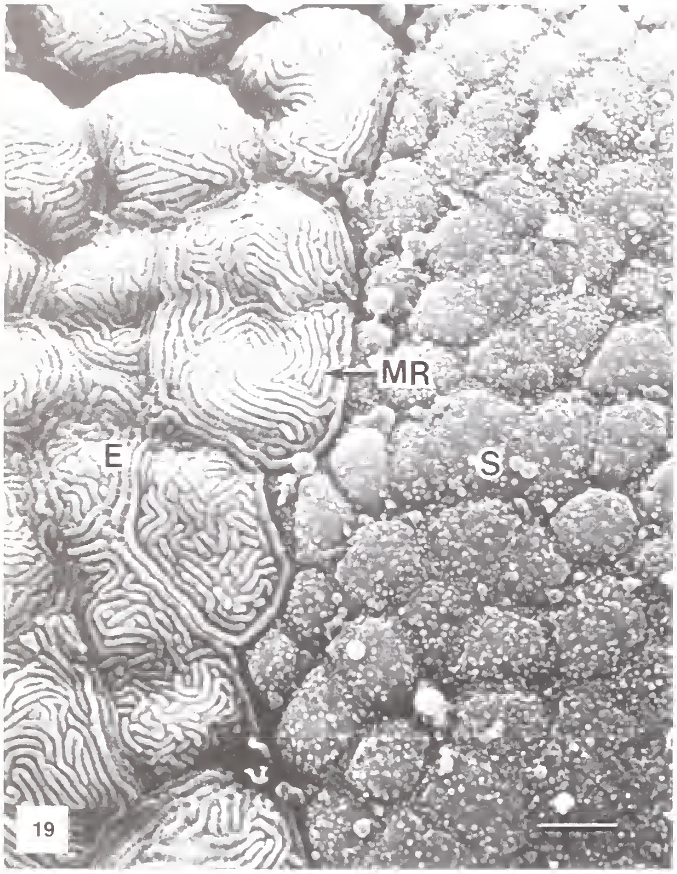

FIGURE 19.—Scanning electron micrograph of apical surface of lining epithelium from northern pike esophageal - stomach junction. An abrupt change from microridge pattern (MR) of the esophagus (E) to the bleblike structures of the stomach (S) is shown. Fixation as in figure 2 legend. Following dehydration, and prior to viewing, tissues were critical point dried and evaporation coated with carbon and gold palladium. Bar= 10 μm. × 1,000

vations of fish skin was reviewed by Henrikson and Matoltsy (*87*). From these observations, it is known that teleost epidermis is a stratified squamous epithelium formed by small basophilic cells and various types of unicellular glands, including mucous cells and club cells (fig. 28). These cells appear to be similar in most species of teleosts, whereas the relative number of each type may vary in localized areas of the skin (fig. 29). Although mammalian skin can be subdivided into several layers, general agreement exists concerning the lack of such stratification in fish epidermis (*85, 87*). For working purposes, however, teleost epidermis is usually subdivided into basal, middle, and surface layers (*87*). In the basal layer are filament-containing (*87*) or Malpighian (*85*) cells; these are low columnar to cuboidal cells with their long axes perpendicular to the underlying dermis (figs. 30, 31). The cytoplasm of these cells is subdivided into perinuclear and peripheral zones (*88*). The perinuclear zone, usually restricted to the area immediately surrounding the nucleus, contains most of the cellular organelles, including mitochondria, granular endoplasmic reticulum, and occasional Golgi profiles. Most of the peripheral zone consists of dense accumulations of tonofilaments approximately 7 nm in diameter and of variable length (*87, 88*). These tonofilaments can be seen in figure 31. Extensive interdigitations of plasma membranes of filament cells are commonly seen, especially in the basal layers of the epidermis (figs. 30, 31). As one progresses from basal to surface regions, cells usually show flattening to various degrees as they assume a horizontal position (*85*). In addition, small microvillus-like projections appear on the apical surfaces of these cells as they approach the outermost epithelial layer (fig. 32). Scanning electron microscopy has demonstrated a correlation between these projections and the microridge pattern seen on the surface cells. Although patches of keratinized epithelium can be found in certain teleosts (*89–91*), this is not a general feature of teleost epidermis. In addition to the above mentioned filament-containing cells, other types of cells include: mucous, club, chloride, granule, and various sensory cells primarily concerned with the sensations of taste and other forms of chemoreception.

In studies with both light and electron microscopy, workers commonly agree that recognizable mucous cells are found primarily in the intermediate and surface layers of teleost epidermis (*89, 92, 93*). In investigations of salmonid fishes (*88*), mucous cells are difficult for one to distinguish from filament-containing cells and cannot be recognized in the basal layers of the epidermis. Similar observations have been reported in other studies of teleost epidermis (*85*). The ultrastructural components of fish mucous cells are similar to those of mammalian goblet cells. They usually appear as rounded-to-ovoid cells and in the most mature forms contain abundant packets of mucosubstance in the apical portion of the cell which is surrounded by an electron dense zone of variable thickness (figs. 32, 33). In mature mucous cells, the nucleus is compressed against the basal cell membrane, where it occurs in close association with the Golgi complex and granular endoplasmic reticulum (*94*). Occasionally, immature mucous cells containing only single or a few small mucous granules may be observed in the deeper zones of the intermediate layer.

Another cell type, the club cell, is restricted to the intermediate zone of the skin (*85*). Club cells in some teleost species secrete a pheromone-like "alarm substance" that elicits a fright reaction in nearby fish (*95*). Specialized club cells responsible for this type of secretion are more appropriately designated as "alarm substance cells" and are shown in figures 28–30 and 33. The typical identifying features of club cells include: large size, centrally placed nuclei (frequently binucleated), and a large zone of fibrillar material characterized by a low electron density (*85, 96*). Immediately surrounding the nuclei are small fragments of membranes, some of which are granular endoplasmic reticulum. The mitochondria present are frequently swollen, whereas other membrane fragments are often found in a vacuolar type arrangement (*96*). General consensus exists that these cells are unicellular glands, although their

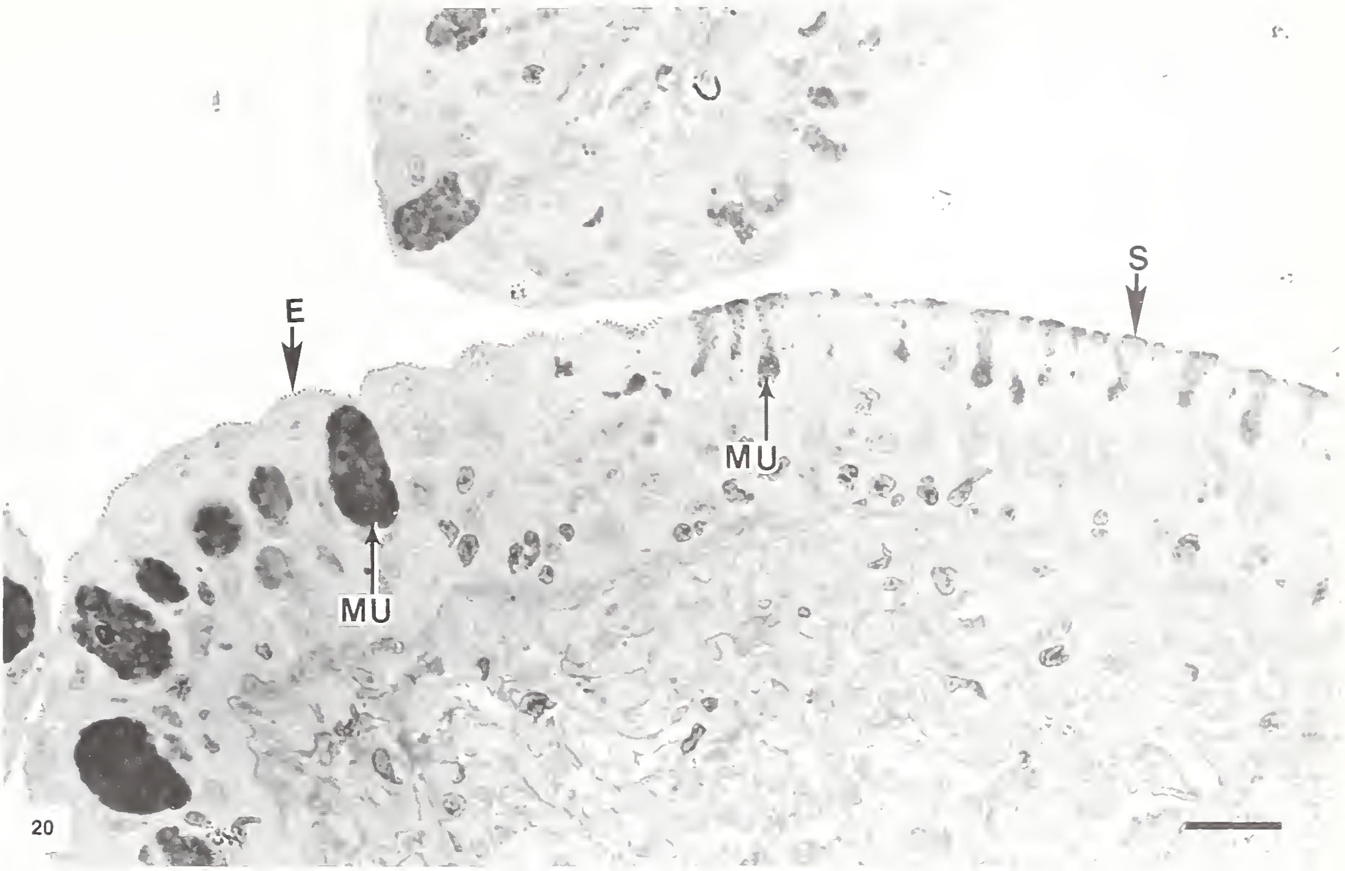

FIGURE 20.—Photomicrograph of a section through Epon-embedded esogaster from northern pike shows the cessation of microridge pattern of esophagus (E) and the alteration in packaging of mucus (MU) seen in mucosa of stomach (S). Fixation as in figure 2 legend. Toluidine blue. Bar=5 μm. × 2,520

function has not been determined, except for those known to secrete the alarm substance (*85, 96*). Blackstock and Pickering (*97*) have described acidophilic granule cells in the epidermis of brown trout; they believe that the proteinaceous secretion of these cells may play a protective role at the epidermal surface. Other types of granular cells have also been noted in teleost epidermis, but their functions remain unknown (*85, 96*). Wandering types of lymphocytes and some macrophages can be found occasionally in teleost epidermis (*85*). Lymphocytes appear to be most common in intercellular spaces of the basal zone, and some workers have attributed this localized finding to a lymphatic-like space (*91*). Detailed ultrastructural observations of these cell types are in the literature (*87, 88, 92, 93, 96, 98–101*).

Few studies have involved differentiation of the above cell types in fish skin. However, Downing and Novales (*102*) have suggested a generalized scheme for vertebrate epidermis in which a relatively undifferentiated cell type gives rise to basic cell types normally found in the epidermis of each respective vertebrate group. Recent work by Zuchelkowski and Lantz (*103*) indicates that both filaments and small mucous granules occur in the same cell, which suggests that the filament-containing cells are the stem cells for both the nonmucous filament-containing and mucous types. In addition, morphometric analysis of brown bullhead (*I. nebulosus*) epidermis has shown that, under conditions of acid stress, mucous volume density increased significantly (figs. 34, 35) due to hyperplasia of mucous cells (*104*). Although use of tritiated thymidine to label S-phase cells indicates that progenitor cells are found in all zones of teleost epidermis (*105, 106*), no increase in mitotic activity was observed in acid-stressed epidermis. Thus it appears that the increased number of mucous cells may arise from differentiation of a preexisting type, i.e., the filament-containing cells.

The next layer for consideration is the dermis, which is usually divided into 2 layers, the "stratum spongiosum" and the "stratum compactum (*85*)." The upper layer, the stratum spongiosum, consists of a collagenous connective tissue stroma which supports various cellular components, including mast cells, mononuclear cells, and various types of chromatophores. In scaled species, the cells responsible for scale formation are also located in this layer. The stratum compactum primarily consists of dense bundles of collagen fibers arranged in a series of layers at right angles

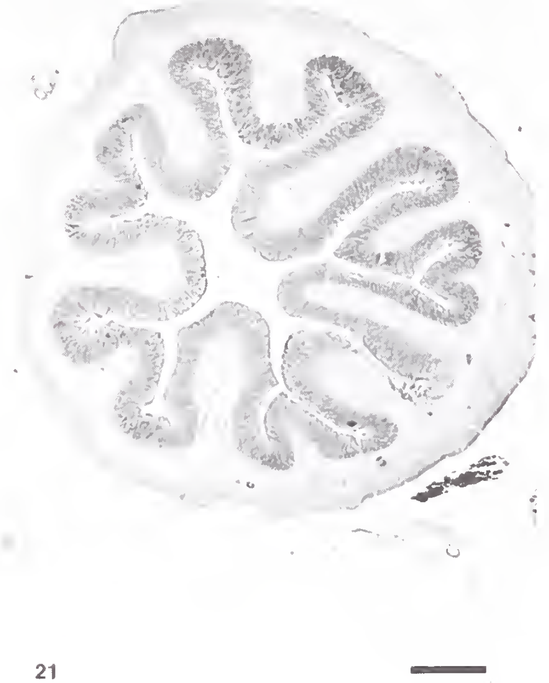

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FIGURE 21.—Paraffin section of corpus of an empty northern pike stomach. Fixation was in Bouin's fluid. H & E. Bar= 200 μm. × 50

to each other. Blood vessels and nerves pierce the collagen bundles at regular intervals. Cell types present include fibroblasts, a few mast cells, and melanocytes (*85*). The components of the scale have been reviewed by Van Oosten (*89*). The typical ctenoid scale consists of a calcified surface layer surrounding a deeper fibrous section commonly referred to as the fibrillary or basal plate. The thickness of the epidermis beneath the scale is greatly decreased compared with the epidermis on the outer surface of the scale.

TELEOST NEPHROBLASTOMAS AND NEPHROGENESIS

Nephroblastomas have been reported in rainbow trout (*Salmo gairdneri*) after dietary exposure of adult fish to dimethylnitrosamine (*14*), exposure of embryos to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (*15*) and after intragastric insertion of the latter compound in adult fish (*107*). Hendricks et al. (*15*) presented a histological description of the tumor that included epithelial components which were incompletely and imperfectly differentiated basophilic tubules and glomeruli with sparse supportive connective tissue. In an analysis of Japanese medakas of various ages, we noted basophilic tubules and glomeruli in control animals. Because numerous mitotic figures were associated with the parietal layer of Bowman's capsule in these glomeruli and in the S-shaped basophilic tubules, we interpreted these as "forming" nephrons. These forming nephrons appeared to resemble similar structures described in the rat kidney (*108, 109*). Furthermore, we demonstrated continuity between basophilic regions of tubule and acidophilic regions characteristic of mature proximal tubular epithelium. This stimulated our interest in the formation of nephrons with respect to age in teleosts. Hoover (personal communication) indicated that she has observed similar structures in mature fathead minnows. Figure 36 shows a field in which an immature-appearing glomerulus is seen. The glomerular tuft is associated with a cleft-like Bowman's space. Continuity from parietal Bowman's capsule to basophilic tubules was observed; the basophilic tubules in this instance contained definitive lumina. Mitotic activity is demonstrated in a basophilic tubule in close association with an immature-appearing glomerulus (fig. 37). Also shown in figure 37 is a region of continuity between basophilic cells and the acidophilic cells normally associated with mature elements of the proximal tubule. Bonvalet et al. (*110*) studied compensatory renal hypertrophy in young rats and found that unilateral nephrectomy, when performed in animals 30–50 days of age, resulted in an increase in the number of glomeruli in the remaining

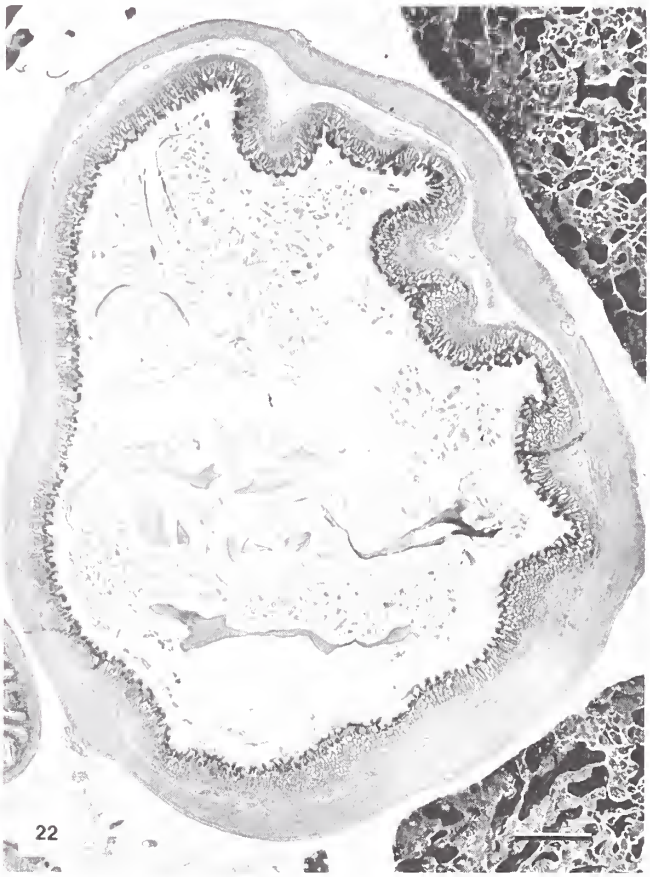


FIGURE 22.—Paraffin section of corpus of a full northern pike stomach. Fixation and staining as in figure 21 legend. H & E. Bar=0.5 mm. × 20

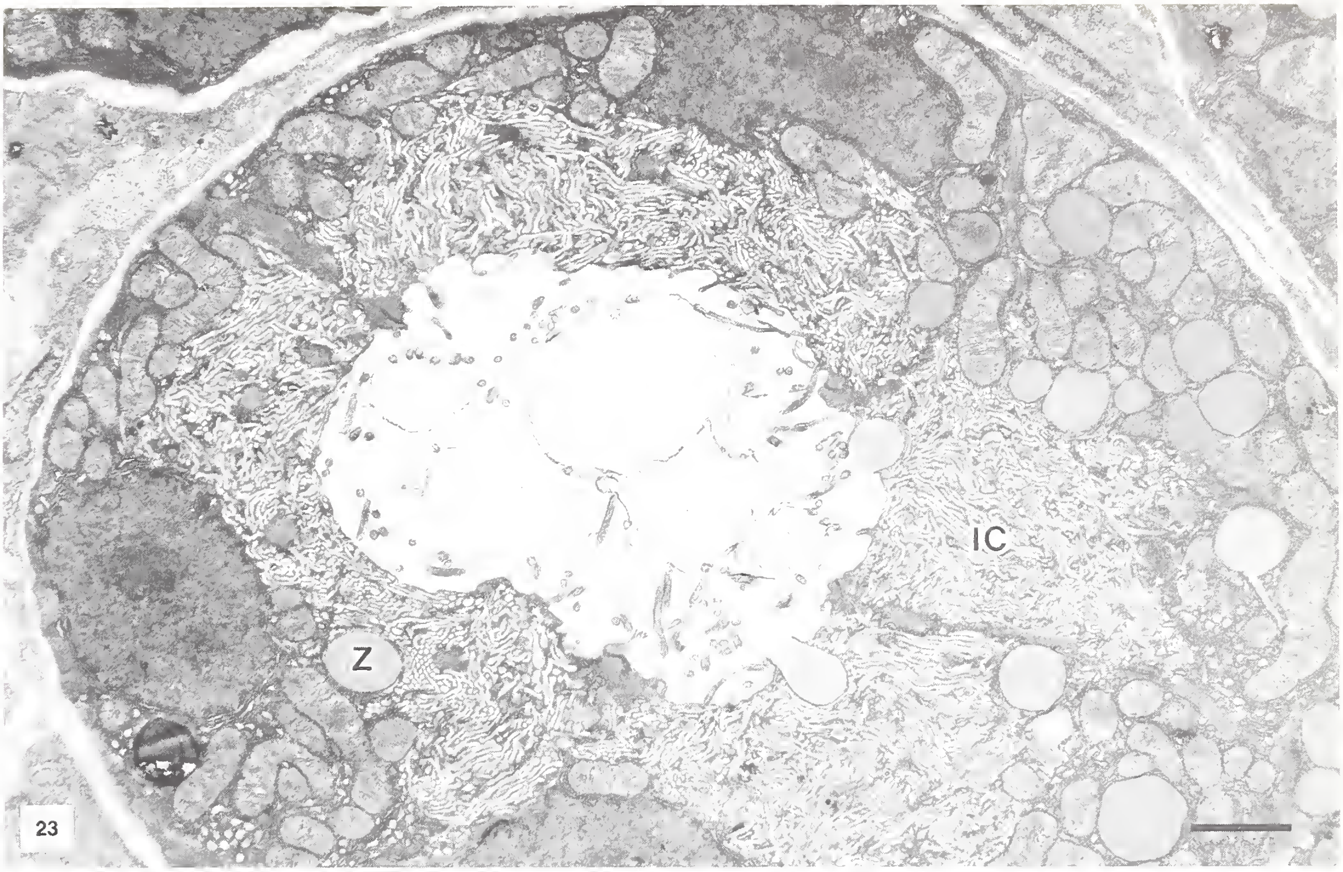


FIGURE 23.—Transmission electron micrograph of a gastric gland from northern pike stomach. The parieto-chief cells contain both intracellular canaliculi (IC) and zymogen granules (Z). Fixation as in figure 2 legend. Uranyl acetate and lead citrate. Bar = 2 μm. × 6,600

kidney. When a similar experiment was performed in rats after 2 months of age, no increase was seen in the number of glomeruli in the remaining kidney. Whether an age-associated loss in the ability to form new nephrons occurs in fishes is not known. Such information would be important in the determination of early lesions which may be difficult to differentiate from normal nephrogenesis.

FIXATION OF TELEOST TISSUES

The increasing use of fish in the study of carcinogenesis makes it imperative that optimal fixation of tissue samples be obtained. For example, in liver carcinogenesis, determination of early lesions is made on the basis of increased basophilia or eosinophilia of cells in H & E-stained sections (*10, 18, 20*), altered glycogen staining (*18*), and presence, absence, or alteration in the amount of histochemical reaction product for specific marker enzymes (*18, 111*). Precise identification of component cell types in tumors may require histochemical, ultrastructural, and immunocytochemical approaches. Thus it is apparent that the use of these diverse methods will place unique demands on the fixation procedure(s). When the limited size of individual organs of small aquarium fishes is considered, the need for careful selection of fixative(s) is even more apparent.

In a review, Hopwood (*112*) aptly stated a basic but often forgotten premise of fixation: "No one fixative is ideal for all situations." Most of the studies on suitability of various fixatives have been on mammalian tissues. For example, formaldehyde, glutaraldehyde, and mixtures of formaldehyde-glutaraldehyde have been studied for their potential usefulness as fixatives for both light and electron microscopy (*113*). A combination of 4% formaldehyde and 1% glutaraldehyde in a 176-milliosmolar phosphate buffer yielded satisfactory results in both standard histological staining of paraffin sections and electron microscopy of plastic-embedded rat and human tissues. However, Davenport and Ball (*114*) found that a similar glutaraldehyde–paraformaldehyde fixative (Karnovsky's) yielded unsatisfactory results with 2 standard connective tissue stains, i.e., Mallory's aniline blue stain for collagen fibers and Gomori's one-step trichrome stain. Puchtler and her co-workers (*115*) studied tissue fixation with Carnoy's fluid or methacarn solution (*116*), i.e., methanol substituted for ethanol in Carnoy's fluid; the latter fixative was considered superior to Carnoy's fluid. To the best of our knowledge, controlled fixation studies of the type just cited (*113–116*) have not been undertaken with fish tissues, and we believe that such studies are overdue. In the fixation of fish tissues, most investigators have used Bouin's fluid (*15, 43, 45, 50, 55, 97,*

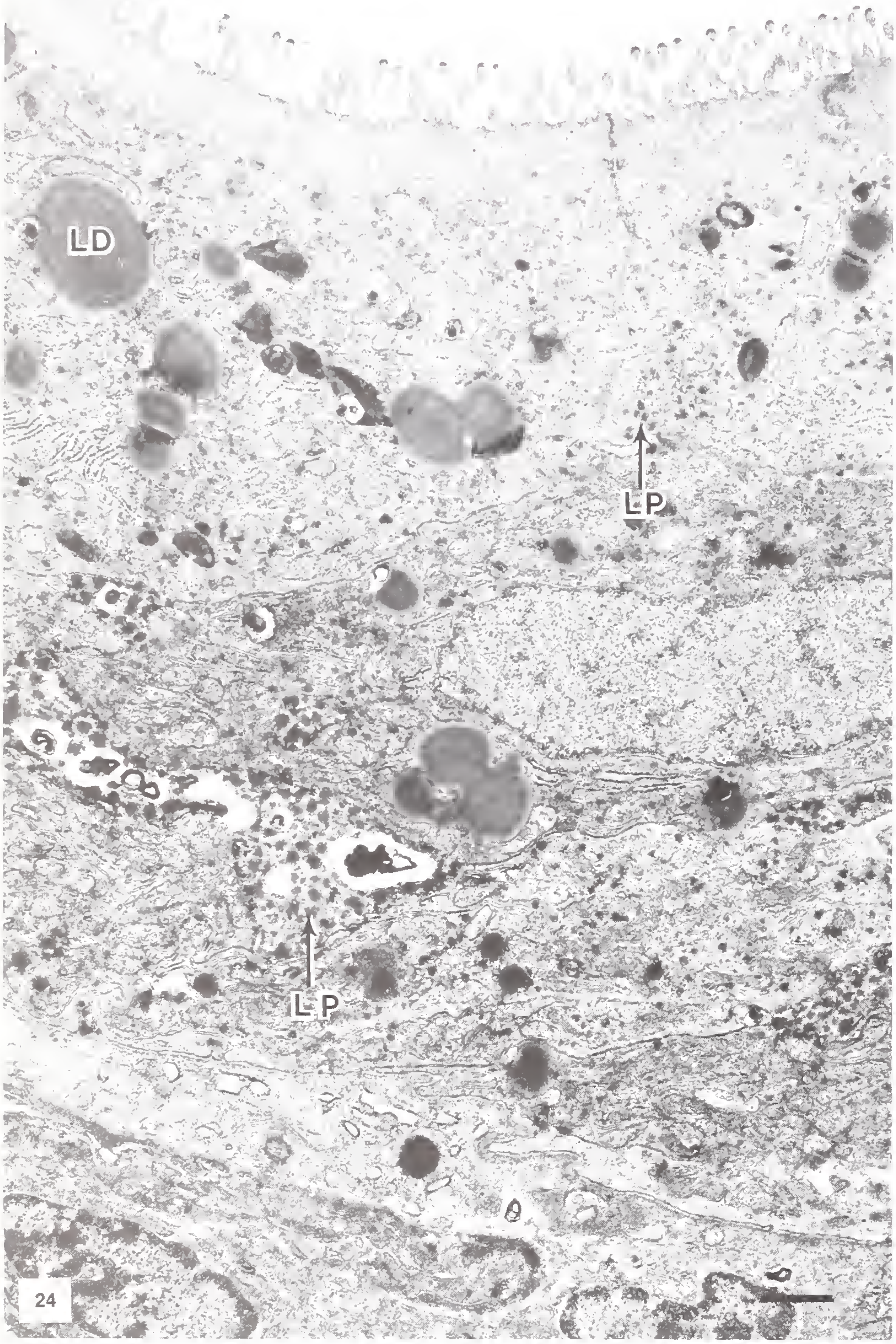
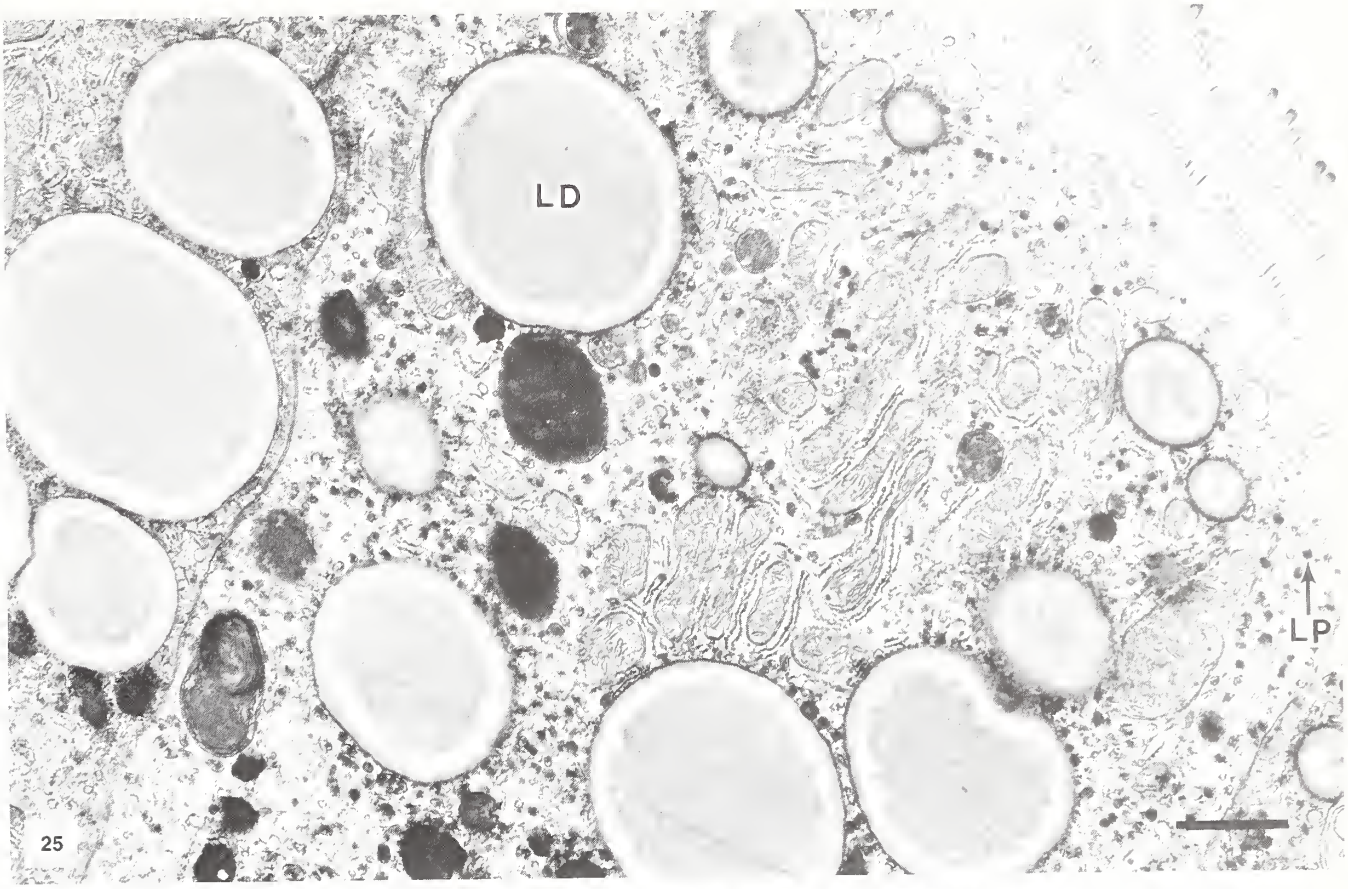
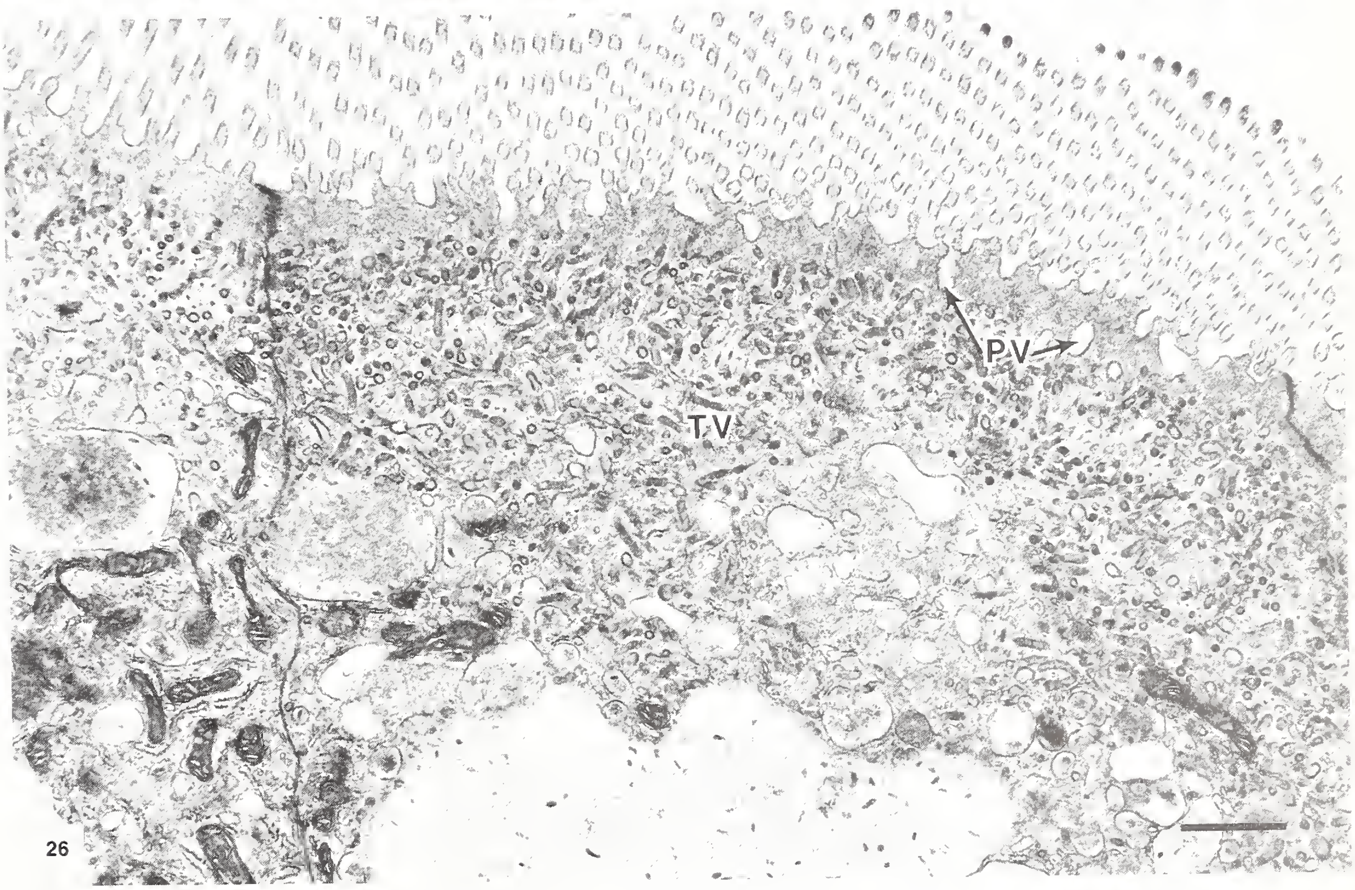


FIGURE 24.—Transmission electron micrograph of a lipid absorptive cell from fathead minnow intestinal bulb. Lipid transport particles (LP) and larger lipid droplets (LD) are seen in these cells. Fixation as in figure 2 legend. Uranyl acetate and lead citrate. Bar=1 μm. × 10,000

FIGURE 25.—Transmission electron micrograph of lipid absorptive cell from walleye intestine. This cell contains lipid transport particles (LP) and large lipid droplets (LD). Fixation as in figure 2 legend. Uranyl acetate and lead citrate. Bar=1 μm. × 14,850

FIGURE 26.—Transmission electron micrograph of protein absorptive cell from walleye intestine. Pinocytotic vesicles (PV) at the bases of the microvilli and the extensive apical tubulo-vesicular network (TV) are characteristics of this cell. Fixation as in figure 2 legend. Uranyl acetate and lead citrate. Bar=1 μm. × 13,750


LD
LP
25


PV
TV
26

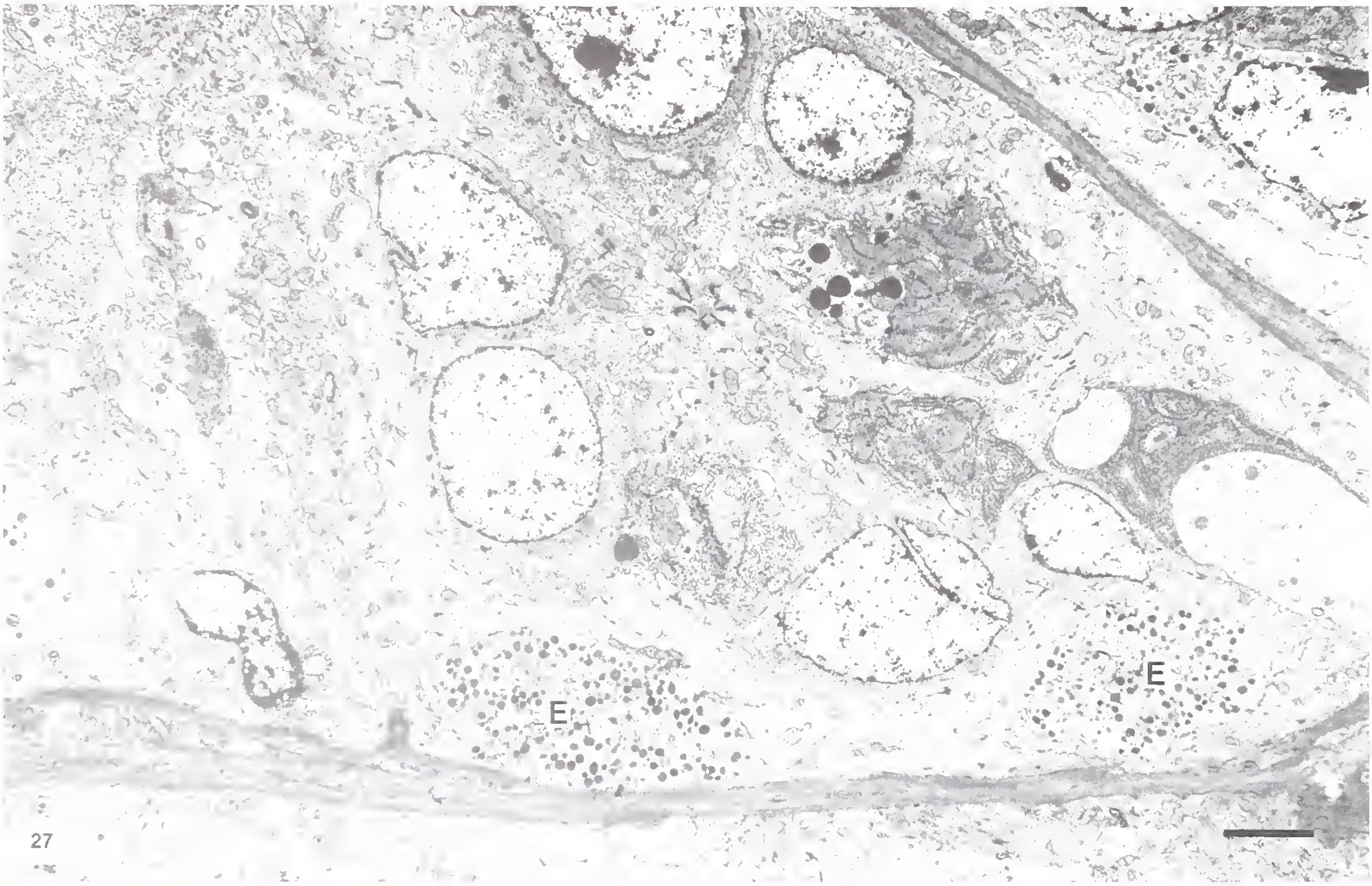


FIGURE 27.—Transmission electron micrograph of walleye stomach wall showing enteroendocrine cells (E) near lamina propria around base of a gastric gland. Fixation as in figure 2 legend. Uranyl acetate and lead citrate. Bar=3 μm. × 4,000

117–122) or 10% neutral buffered formalin (*6, 8, 52, 55, 56, 68, 97, 118, 120, 121, 123*). Decalcification, a well-documented effect of the acetic acid in Bouin's fluid (*124*), undoubtedly plays a role in the popularity of this fixative. Sagittal sections of entire fish are usually obtained following fixation in Bouin's (*5*), and the decalcification effect can be enhanced by substitution of acetic acid with a stronger acid, e.g., formic acid (*7*). Some statements of caution regarding either of these two fixatives should be made: 1) Bouin's fluid is an excellent fixative, but nuclear staining may be poor and this fixative may result in the destruction of some cellular secretory products (*124*); and 2) formalin

TABLE 1.—*Site and histochemistry of mucous cells in mouth and pharynx of American shad*[a]

Reaction	Mouth	Pharynx
Gomori's one-step trichrome	More in roof than floor	Equal in roof and floor
Periodic acid-Schiff	Strongly +	Strongly +
Alcian blue, pH 2.5	Strongly +	Strongly +
Alcian blue, pH 2.5, and periodic acid-Schiff	Most are +; some are only Alcian blue +	Same as mouth
High iron diamine	Roof varies from strongly to weakly +; floor strongly +	Same as mouth
High iron diamine and periodic acid	All cells high iron diamine +	Same as mouth
High iron diamine and Alcian blue	Roof cells Alcian blue + or high iron diamine +; all floor cells high iron diamine +	High iron diamine + (gill rakers and lamellae, roof and floor); Alcian blue + or high iron diamine + (gill arch)

[a] + = positive.

TABLE 2.—*Site and histochemistry of mucous cells in esophagus of American shad*[a]

Reaction	Rostral one-third	Middle one-third	Caudal one-third
Gomori's one-step trichrome	Majority in mucosal folds	Along all of luminal surface	Similar to middle one-third but less cells
Periodic acid-Schiff	Strongly +	Strongly +	Strongly +
Alcian blue, pH 2.5	Strongly +	Strongly +	Negative
Alcian blue, pH 2.5, and periodic acid-Schiff	All + or only Alcian blue +; no periodic acid-Schiff + only	All + with periodic acid-Schiff predominant	Nearly all periodic acid-Schiff +; few Alcian blue + or all +
High iron diamine	Mixed strongly and weakly +	Strongly + (base of folds); weakly + (other sites)	Most cells negative
High iron diamine and periodic acid	All moderately to strongly +	Same	Same
High iron diamine and Alcian blue	Mixed high iron diamine and Alcian blue +	Most Alcian blue +; some high iron diamine +	Same as middle one-third

[a] + = positive.

alone is ". . . one of the poorest preparations for paraffin-embedding (*124*)." Fortunately, most investigators do not use formalin alone, rather they add compounds for buffering the solution, or add sucrose or saline in an effort to improve the quality of final fixation. Pearse (*125*) regards formalin fixation as incomplete until 7 days in the fixation have elapsed. Even though it is generally assumed that formalin does not fix carbohydrates, Spicer and coworkers (*78*), studying the histochemistry of connective tissue glycoconjugates, have stated that formalin-free fixatives were never superior to those with it, and some were in fact inferior. Zaccone (*121*) used formalin–cetylpyridinium chloride in the study of mucins in teleost skin. We found that it yielded excellent results in our laboratory with respect to the preservation of glycoconjugates in various tissue types. This is an excellent fixative for glycoconjugates, particularly those of an acidic nature, and should be more widely studied as a fixative for fish tissues.

Different modifications of an acetic acid-alcohol–formaldehyde solution have been used as fixatives for fish (*66, 126*); solutions of this nature are found in the literature under various names, e.g., as Serra's fixative (*66*), Dietrich's fluid (*126*), or Kahle's fluid (*127*). All these solutions are probably modifications of the acetic acid–alcohol–formaldehyde solution of Tellyesniczky developed in 1898 (*128*). Because of its decalcifying effect, Dietrich's fluid has been recommended as a fixative for fish (*126*). The decalcifying effect of this fixative results from its acetic acid content (Yevich PP: Personal communication). Other fixatives used in fish studies include Carnoy's fluid (*117, 129*), Zenker's fixative (*118, 121*), or Zenker–formalin (Helly's) (*119, 121*). Unfortunately, none of the commonly used fixatives for light microscopy studies of fish tissues yield fixation of the quality necessary for electron microscopy.

The use and suitability of some form of aldehyde fixation followed by postfixation in osmium tetroxide for electron microscopy of fish tissues is well-documented (*26, 29–32, 35, 64, 119, 130, 131*). Consideration of perfusion fixation of whole fish should also be given if one is to obtain what may well be optimal fixation for electron microscopy (*131*). An investigator will find that it is often helpful to have one fixative suitable for paraffin embedding or plastic embedding, or both. We have used the 4% formaldehyde–1% glutaraldehyde fixative which McDowell and Trump (*113*) recommended and have obtained satisfactory ultrastructural morphology. We have also found that, with electron microscopy, examination of fish intestine and liver specimens fixed in 10% buffered formalin allowed confirmatory analysis of the morphology seen at the light microscopic level, but the fixation was clearly not optimal.

Observations on various fish tissues fixed in a variety of fixatives led us to believe that some commonly used fixatives were unsuitable for certain tissue types. As a result, two of us (Hinton DE, Pinkstaff CA) examined coded slides of fathead minnow liver and intestine previously fixed in either Bouin's fluid (*124*), 10% neutral buffered formalin (*132*), Karnovsky's fixative (*133*), Dietrich's fluid

TABLE 3.—*Site and histochemistry of mucous cells in stomach of American shad*[a]

Reaction	Corpus	Pylorus
Gomori's one-step trichrome	Mucus in lining cells apices	Mucus in apices of cells lining gastric pits
Periodic acid-Schiff	Strongly +	Strongly +
Alcian blue, pH 2.5	Negative	Positive
Alcian blue, pH 2.5, and periodic acid-Schiff	Periodic acid-Schiff only	All +
High iron diamine	Negative	Strongly +
High iron diamine and periodic acid	Moderately to strongly +	Moderately to strongly +
High iron diamine and Alcian blue, pH 2.5	Negative	Strongly high iron diamine +

[a] + = positive.

TABLE 4.—*Site and histochemistry of mucous cells in intestinal ceca and intestine of American shad*[a]

Reaction	Intestinal ceca	Intestine
Gomori's one-step trichrome	True goblet cells scattered in mucosa	Same with increased number distally
Periodic acid-Schiff	Strongly +	Same
Alcian blue, pH 2.5	Strongly +	Same
Alcian blue, pH 2.5, and periodic acid-Schiff	All + or only Alcian blue +; no periodic acid-Schiff + only	Same
High iron diamine	Most goblet cells strongly +	No data
High iron diamine and periodic acid	Moderately to strongly +	No data
High iron diamine and Alcian blue	Strongly high iron diamine +	Some high iron diamind +, majority Alcian blue +

[a] + = positive.

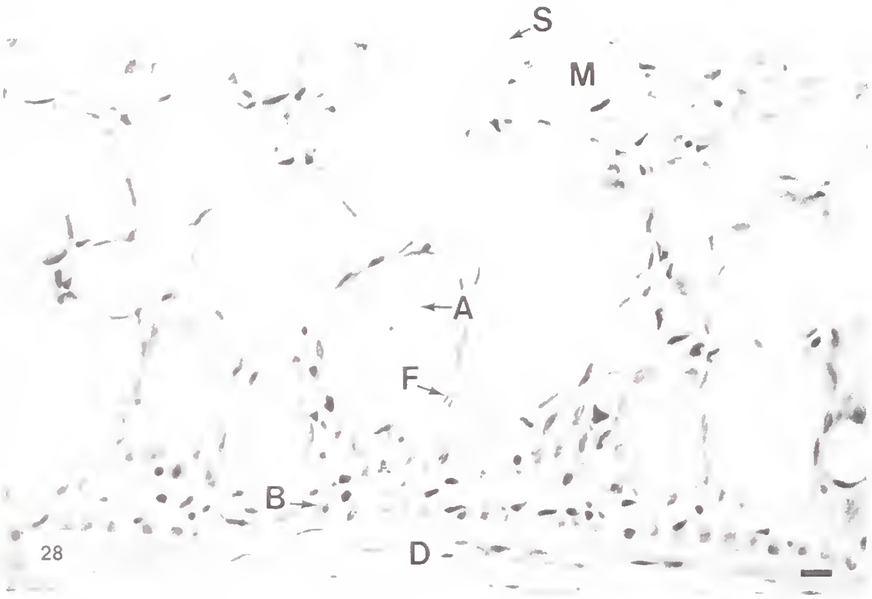


FIGURE 28.—Photomicrograph of paraffin section through epidermis of a nonscaled teleost, the brown bullhead catfish. A layer of flattened epithelial cells (S) is seen at the surface. Large, pale-staining alarm substance cells (A), mucous cells (M), and filament-containing cells (F) occupy the midepidermal region. A single layer of basal cells (B) lies immediately above the dermis (D). Fixation in 10% unbuffered formalin–1% cetylpyridinium chloride. H & E. Bar = 10 µm. × 380

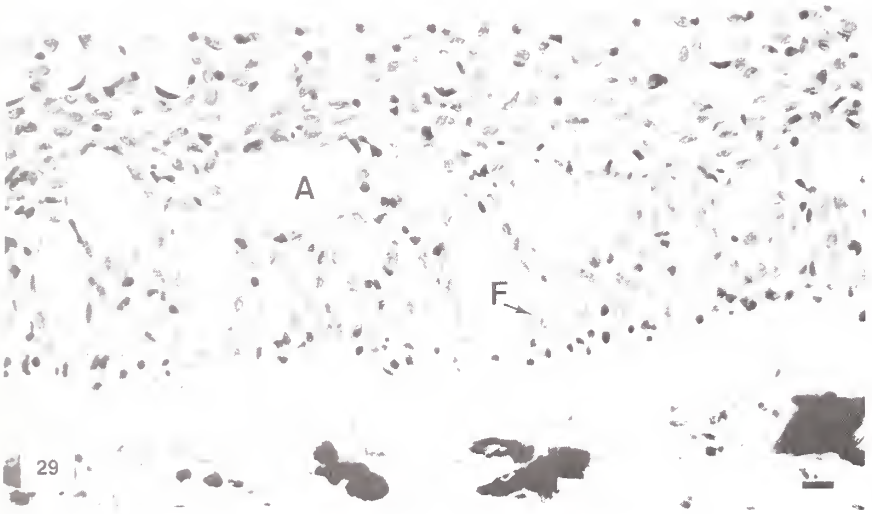
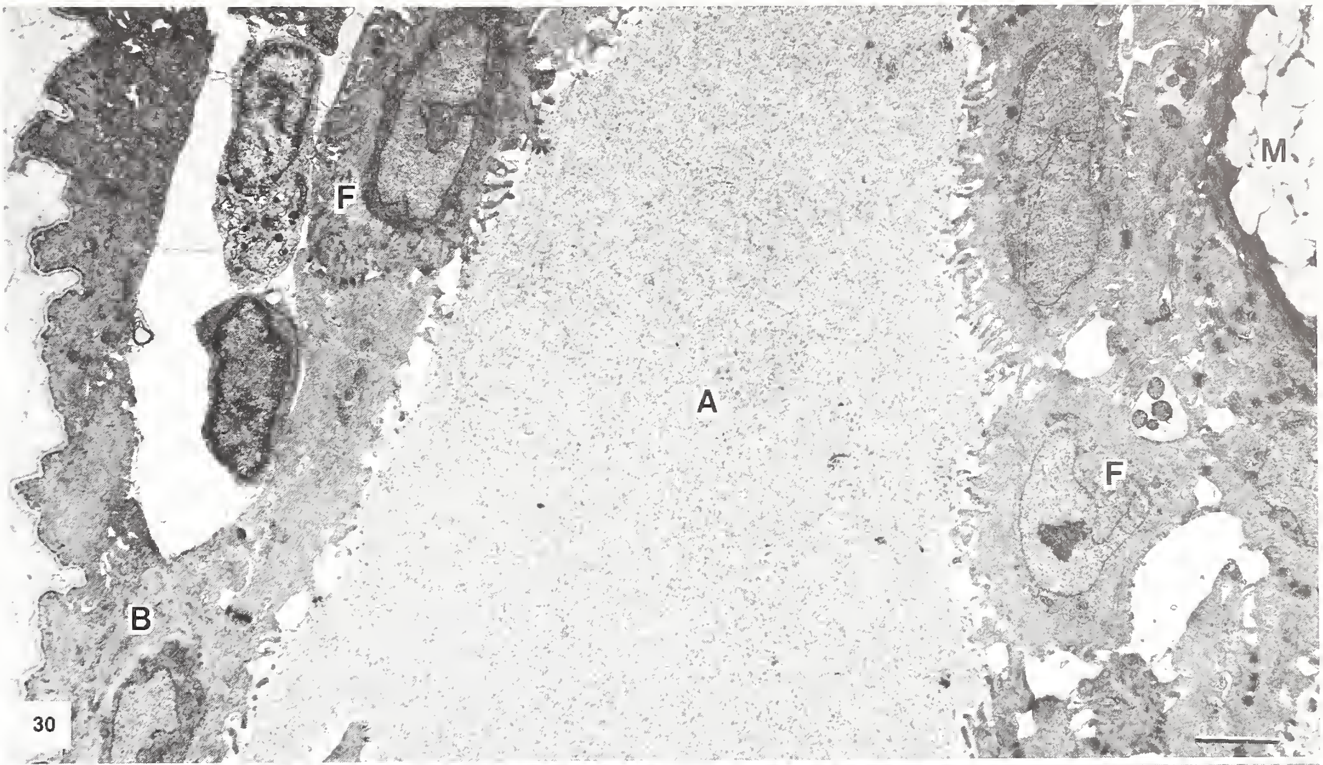


FIGURE 29.—Section of epidermis of the fathead minnow showing cell types similar to those in figure 28, although more basophilic cells [possibly filament-containing cells (F)] and fewer alarm substance cells (A) are seen. Fixation as in figure 28 legend. H & E. Bar = 10 µm. × 380



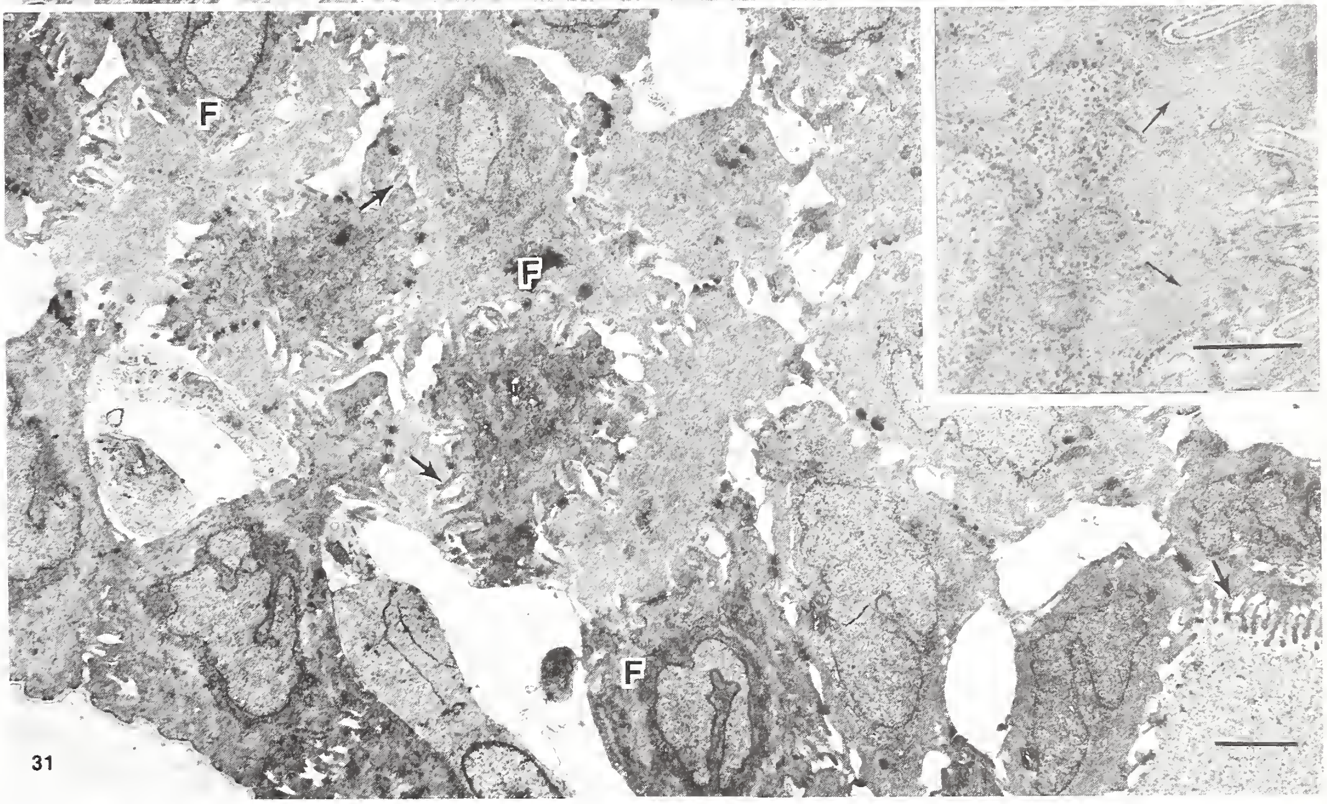
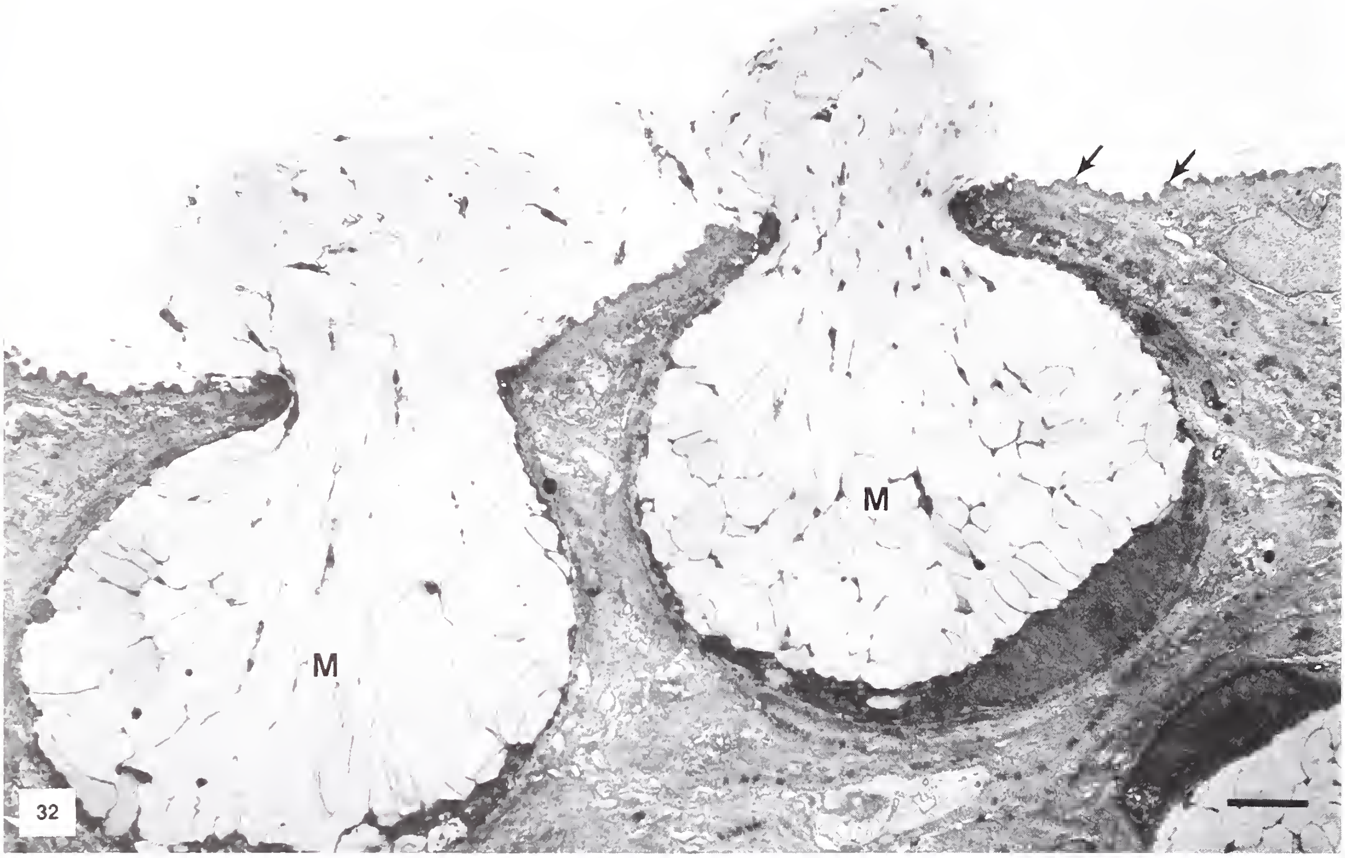
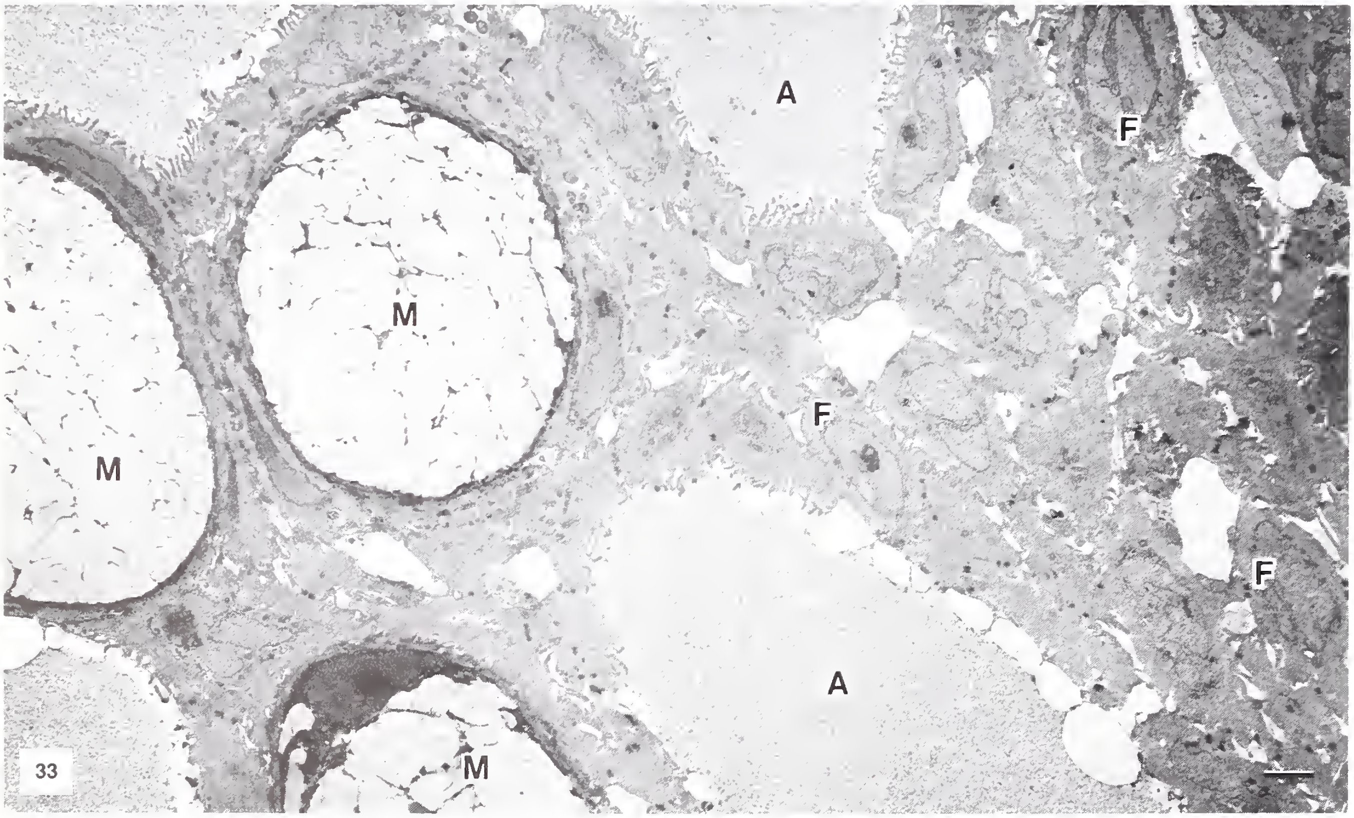
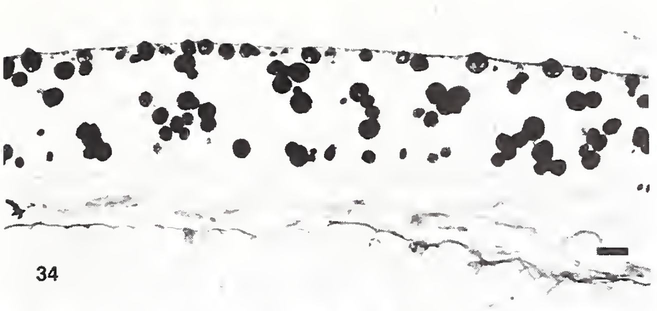


FIGURE 30.—Transmission electron micrograph of basal region of brown bullhead epidermis demonstrating a single layer of basal cells (B) adjacent to the basement membrane at far left of figure. Portions of an alarm substance cell (A) and a mucous cell (M) are also present with numerous filament-containing cells (F). Nonepidermal, wandering cells are visible in the intercellular spaces to the right of the basal layer. Fixation in 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.2). Uranyl acetate and lead citrate. Bar = 2 μm. × 5,500

FIGURE 31.—Transmission electron micrograph of region adjacent to figure 30 exhibiting an abundance of filament-containing cells (F). Extensive interdigitations occur between adjacent filament-containing cells and between filament-containing cells and alarm substance cells (*arrows*). Fixation as in figure 30 legend. Bar = 2 μm. × 5,500. *Insert:* Portion of a filament cell, showing bundles of tonofilaments in the peripheral zone of the cytoplasm (*arrows*). Bar = 0.5 μm. × 28,000


M
M
32


A
F
M
F
M
F
A
M
33



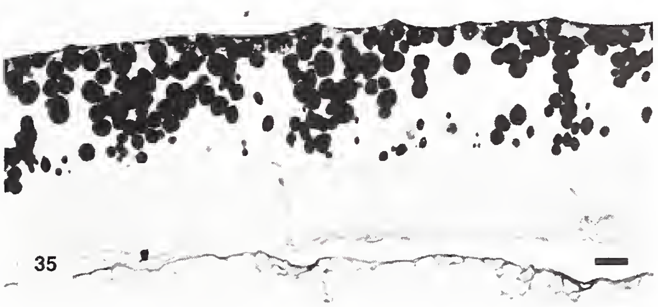


FIGURE 34.—Photomicrograph of paraffin section from control brown bullhead epidermis reacted for acidic and neutral mucosubstances. Fixation in 10% unbuffered formalin–1% cetylpyridinium chloride. Alcian blue (pH 2.5)–periodic acid-Schiff's reagent. Bar=40 μm. × 100

FIGURE 35.—Photomicrograph of paraffin section through epidermis of acid-stressed brown bullhead catfish. The number of cells reacting positively for acidic and neutral mucosubstances is greatly increased when compared with control epidermis (fig. 34). Fixation as in figure 34 legend. Alcian blue (pH 2.5)–periodic acid-Schiff's reagent. Bar=40 μm. × 100

(*127*), or Davidson's fluid (*134*). Sections of tissues fixed in the above solutions were stained with H & E. In addition, various glycoconjugate-staining techniques recommended by Spicer and co-workers (*78*) were used. These included: periodic acid-Schiff, Alcian blue at pH 2.5, periodic acid-phenylhydrazine-Schiff, combined Alcian blue–periodic acid-Schiff, and high-iron diamine–Alcian blue.

Examination of tissues showed that Bouin's and Davidson's fluids and 10% neutral buffered formalin were most effective in maintaining morphological appearance at the light microscopic level. Karnovsky's fluid was slightly less effective and Dietrich's fixative was the least effective. The preservation of connective tissue morphology was extremely poor in tissues fixed in Dietrich's fluid, particularly the lamina propria of the small intestine which showed considerable shrinkage. Staining of the glycoconjugates in goblet cells of the small intestine was excellent with all fixatives. Tissues fixed in 10% neutral buffered formalin or Karnovsky's fixative exhibited an overall periodic acid-Schiff staining of smooth muscle, connective tissue, and epithelial cells which may well have been due to the presence of preexisting aldehydes. It may be necessary to block such preexisting aldehydes before periodic acid-Schiff staining if one chooses to use either of the latter 2 fixatives or any other aldehyde fixatives. Such blockage can be achieved by pretreatment of sections with either dimedone or borohydride (*135*).

Both with respect to the intensity of staining by the periodic acid-Schiff technique and to localization of liver glycogen, results were dependent on the fixative used. Bouin's and Davidson's fluids gave excellent results in regard to uniformity of stain within hepatocytes and throughout the liver. No "streaming artifact" of glycogen was noted when these fixatives were used. No streaming artifact of glycogen was noted in either 10% neutral buffered formalin or Karnovsky's fixed liver; however, the intensity of stain in sections from blocks fixed in these solutions may reflect both glycogen and free aldehyde content. Blocks of liver tissue fixed in Dietrich's fluid were unsuitable for the histochemical demonstration of glycogen. Glycogen was present only in a peripheral zone of the blocks extending only a few cells deep. It was startling for us to observe the differences in both morphological appearance of tissues and their glycogen staining in sections from blocks fixed with Davidson's fluid when compared with the poor results with Dietrich's fluid. Apparently, the addition of glycerol to an acetic acid–alcohol–formaldehyde solution (Davidson's fluid) vastly improves the results. The findings of our pilot study clearly show the need for additional clearly defined studies of controlled fixation in fish tissues.

From the above preliminary results, it is clear that one should carefully consider the method of fixation to be used in fish studies. Dawson (*136*) recommended that human surgical biopsy specimens be divided, size permitting, into 3 pieces; the first sample to be fixed for standard paraffin embedding and subsequent histological staining, a second sample to be quenched (rapidly frozen at low temperature, below −70° C), and a third to be fixed in an aldehyde fixative for later examination by electron microscopy. Investigators working with fish tissues might wish to adopt such a plan of tissue preparation that provides subsequent access to tissue for nearly any research methodology.

FIGURE 32.—Transmission electron micrograph of mucous cells (M) discharging at epidermal surface in a brown bullhead catfish. Frequent desmosomal contacts occur between filament-containing cells, and a decreased amount of intercellular space is seen at this level of epidermis. Microridges are noted on filament cells of the outermost epithelial layer (*arrows*). Fixation as in figure 30 legend. Uranyl acetate and lead nitrate. Bar=2 μm. × 5,500

FIGURE 33.—Transmission electron micrograph of midepidermal region in a brown bullhead catfish demonstrates several mucous cells (M), portions of alarm substance cells (A), and numerous filament-containing cells (F). Fixation as in figure 30 legend. Uranyl acetate and lead citrate. Bar=2 μm. × 3,400

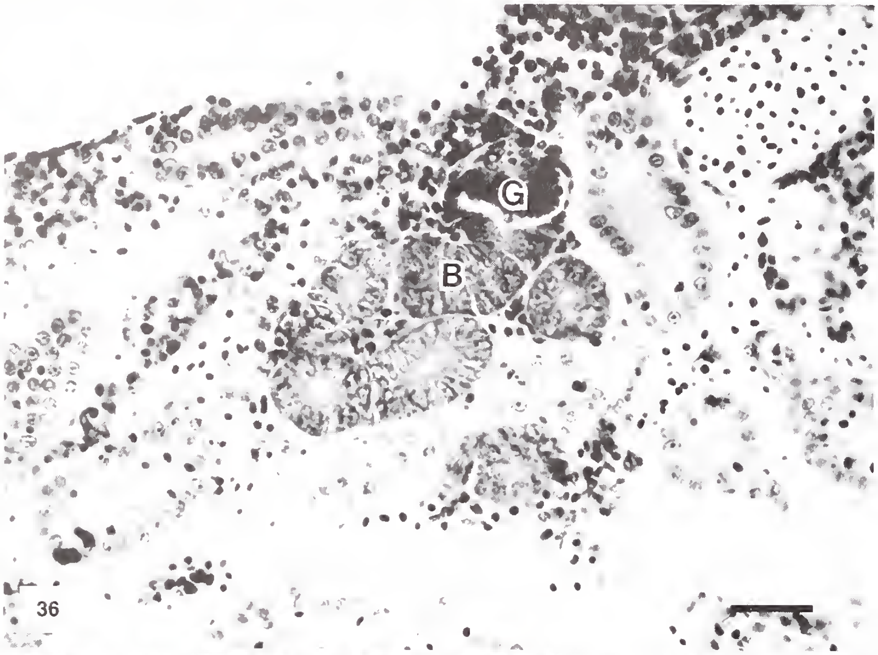


FIGURE 36.—Photomicrograph of paraffin section through fathead minnow kidney shows an immature glomerulus (G) which is continuous with basophilic tubular profiles (B). Fixation in Bouin's solution. H & E. Bar=30 μm. × 360

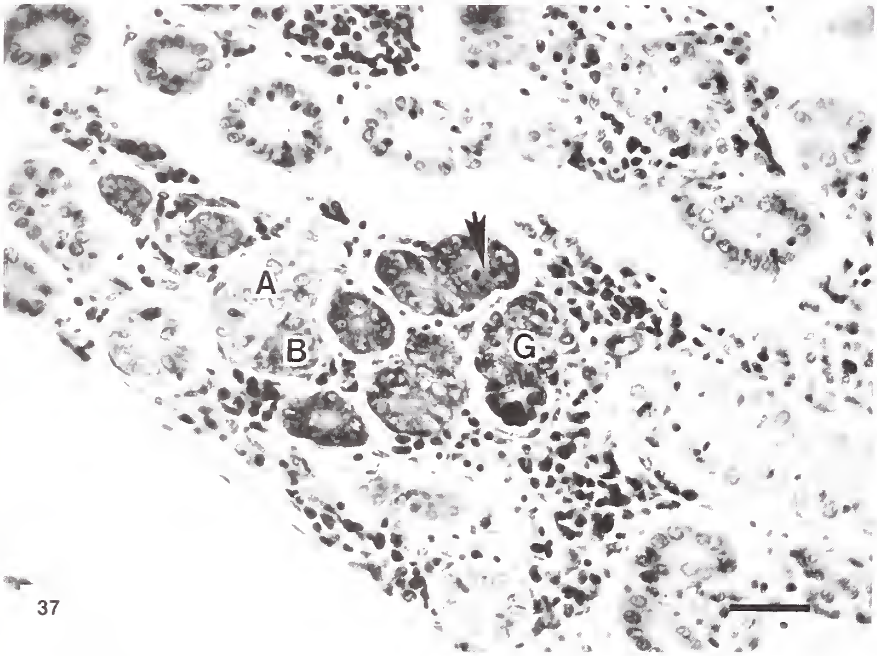


FIGURE 37.—Photomicrograph of paraffin section through fathead minnow kidney. A definitive Bowman's capsule surrounds the immature glomerulus (G). A mitotic figure (*arrow*) is seen in the basophilic tubule. *Note* continuity between acidophilic (A) and basophilic (B) cells. Fixation in Bouin's solution. H & E. Bar=30 μm. × 360

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DISCUSSION

J. M. Rice: Thank you, Dr. Hinton, for a well-illustrated talk. Are there questions for Dr. Hinton?

M. Sigel: I want to second your comment. That was an excellent presentation and anatomically and intellectually stimulating. I learned a good deal. It is exciting to know that as we make inroads into understanding some of the problems of the physiology, immunology, and oncology of fish, we perhaps are also taking a backward look at the anatomy of fish, so that we learn more about the structures, which, as far as I am concerned, have been neither well understood nor defined in the past.

I want to ask you one question. Have you been able to demonstrate any changes in lymphocytes in the skin of fish exposed to your various stresses because, as we have demonstrated before in fish, IgM is made in the skin for secretion. Is there any change in the situation?

D. E. Hinton: We have not quantified this by morphometry, but we see an enhanced space between cells in the basal region that some workers are beginning to think might be a typical lymphatic type of space. We see wandering cells occasionally. It has not been our interpretation so far that we see any greater number of these, but we have primarily been looking at mucosubstance-containing cells. I want to do more with the immune defense system, morphologically, in fish.

Rice: Dr. Martin?

B. J. Martin: Dr. Hinton, you referred to the washed-out appearance of the livers with the different species, and I am aware that that is often related to nutrition. Do you ever see, in a species that you studied, a mosaic pattern in which you find large areas that have this appearance and then other areas that are chromophilic and look normal? If so, what significance do you attach to that observation?

Hinton: Do you see these latter-described areas at the periphery of the sections or blocks?

Martin: No, they may cover large areas, maybe 50%. A well-defined random line is between these two areas.

Hinton: I looked in the literature for any mention of lobular and/or intralobular patterns in fish livers. I think they do not exist; we do not see them ultrastructurally. We have not done histochemical observations, but I think you may be looking at some populations of hepatocytes that are reacting differently or in a different stage of development.

B. Trump: Dr. Hinton, in how many species have you found evidence of tubules forming?

Hinton: We have seen them in the medaka, and we are now going back and looking at them from earlier periods. The ones I showed you were from adult fathead minnows that Dr. Hoover sent me. I have gone back to our fathead minnows and we see them there. So I think that, as in contradistinction to the mammal, possibly this system can continue to make new glomeruli and tubules.

K. Hoover: Dr. Hinton, why do you think that we fail to see more gill tumors in fish? Is there a protective role of the mucus? Do you think that it really is an effective barrier or is it a concentrator of pollutants?

Hinton: We see a lot of mitotic activity, relatively speaking, of interlamellar sites, so certainly the cells are turning over; I would say a fair amount of cell turnover is taking place.

That has intrigued me. I know there is P-450 activity in the gills. Dr. Stegeman can say more about that. I have been surprised that we do not see any more tumors, considering the high turnover rate of cells on the lamellar and arch surface.

J. Lech: What is the configuration of the bile ductules as they enter the gastrointestinal tract in your species? I mention this because some time ago, some people from the Environmental Protection Agency (whose names I cannot recall) indicated that they often see lesions in that area of the intestinal tract in fish exposed to certain kinds of chemicals, and, because the gall bladder concentrates them, they could be excreted in high local concentrations. Have you observed anything like that?

Hinton: In some species, we see a papilla, a finger-like process on the wall, through which this opening passes. I now want to do some studies to try to understand if the "plumbing" is common at that point for both pancreatic and biliary secretions. I have tried to do serial sections. I think I have shown some pancreatic ducts coming in to the bile ducts, but surely you could get a high dose of localized exposure at a site like that. Usually, it is a papilla with the cells around it. There will be some elevation from the wall, normally, and you may or may not see differences there, relative to the rest of the intestine.

C. J. Dawe: I have a couple of questions, and one comment. First of all, I would like to say that I think your work is meticulous and well done, and we need more of that kind of excellence.

My first question is: Is it known whether the increase of mucin in the skin of the female medaka is related to prolactin in the human female? Prolactin does exist in fish even though they do not have mammary glands. It sometimes serves the purpose of stimulating mucus secretion to the extent that it is used for feeding of the offspring.

Hinton: Our observations on that particular aspect apply to the bullhead, not the medaka. I do not know if such a relationship exists, but we are looking for any kind of explanation because these were sexually immature fish. Hayashi has shown in the tracheobronchial system of the rat that the female has more resting mucosubstance in tracheobronchial epithelium. Yes, we are interested in this, but I have no explanation right now. I would like to do further studies on that.

Dawe: Has the embryology of the fish liver been investigated? The question I am interested in is whether the hepatocyte itself does have a different origin. Does it come from mesenchyme, which Elias has claimed and which I doubt, or from the intrahepatic and extrahepatic duct system, which is presumably from the diverticulum?

Hinton: Yes. Based on my understanding, the hepatocytes and all of the epithelium associated with the bile drainage are from the diverticulum of the foregut. The blood elements, connective tissue, and capsule are from the mesenchyme in the septum transversum.

We are most anxious now that we have these breeding populations of medaka to look at just that. I think it is an important question, now that we are exposing embryos, that we have a base line established at all age points in this important fish.

Dawe: May I make the comment that relates to some things that Dr. Rice said? The existence of tubulogenesis in adult fish in the kidney is interesting. We have noticed it, and I am sure lots of other people have. I wonder if it at least partially explains what I believe is true, i.e., that the nephroblastomas, at least in the rainbow trout that we have seen, are almost all well-differentiated nephroblastomas, and rarely, if ever, metastasized, which makes them quite a different beast from what we see in many of the mammals. It looks as if perhaps the ability to differentiate in the adult may have an influence on the quiet nature of the tumors that result.

A. Woodhead: We are interested in the movement of particulates through the gut, and it seems in rats that carbon enters through Peyer's patches and nowhere else. The rest is an effective barrier. We looked for these in the Amazon molly, and I can find nothing equivalent to these patches. Have you seen anything like that in any of these species?

Hinton: No, we have not, and we have looked for them. We are interested in passage of coal particulates. We have looked for Peyer's patches and have not found any focal accumulations. Occasionally in the lamina propria, you see more small accumulations of cells, but nothing like a Peyer's patch.

J. Bend: The responses you show in proliferation of the rough endoplasmic reticulum after Aroclor induction are most impressive. What is the dose and what is the time for response?

Hinton: One polychlorinated biphenyl-impregnated gelatin capsule was placed in the stomach. The dose was 1,000 mg/kg body weight and the time was 21 days.

Bend: Have you coursed it to get the disappearance time?

Hinton: No.

M. Lipsky: We saw some focal vacuolar-type changes, and then at 21 days after exposure we began to see this whorl-type pattern.

Bend: Biochemically, those responses will still be seen after 90 days, and that is what I am interested in, the endoplasmic reticulum response.

Hinton: I have a student now who is doing a biochemical study in the rainbow trout with 3-methylcholanthrene, coupled with the morphometry of the changes in the ultrastructure of the liver cells. We are hoping that we can shed more light on that.

Bend: Do you see a proliferative response like that with polycyclic hydrocarbons, such as B-naphthylflavone and 3-methylcholanthrene?

Hinton: With 3-methylcholanthrene, we get some circular-type profiles, but we have not seen the whorl patterns.

J. A. Couch: We have published at least 1 paper on this with 3-methylcholanthrene and benzo[*a*]pyrene and we see tremendous proliferation of the rough endoplasmic reticulum. You and I have discussed the apparent quantitative absence of smooth endoplasmic reticulum in fish hepatocytes, as opposed to mammalian hepatocytes. They seem to have a lot of the rough type, and that is the component that proliferates when they are exposed to polynuclear aromatic hydrocarbons. We have also found, with Dr. Shore, that the mixed function oxidase system is enhanced at this time. The enzyme activity is increased and induced considerably with this proliferation.

J. Hendricks: Dr. Hinton, I was really impressed by your paper, and I would like to make an observation and ask you a question relative to it.

I have noticed that bile in the rainbow trout, anyway, is extremely necrogenic to liver tissue if topically exposed. Also, we frequently notice that if there is a breakdown of the bile ductule within the liver that the bile leaks out, and, again, we see necrosis of the liver tissue. I wonder if you have seen that sort of thing.

I have another question along that line: For curiosity's sake, we exposed some of the intestinal tract to some collected bile for a period and found that it too was necrogenic to the epithelium of the intestine. What protects the liver cells along the bile canaliculus from that bile? Is there a difference in the bile at that point from that in the gall bladder? What are your comments on those observations?

Hinton: Well, it should be inactive. While you were talking, I could not help but think about the situation in the pancreas because what I wanted to do is to see if I could induce fatty necrosis of the liver because of the association of these exocrine pancreatic cells and hepatocytes. I want to work out how this system drains. Except in acutely damaged livers from methyl mercuric chloride (we did studies with really heavy ip injections), we have not seen this breakdown of the ductular system. In those in which we did, we had gross changes with marked necrotic regions.

P. Weiss: First I have also seen the immature kidney tubules in *Fundulus heteroclitus,* but I think that is not surprising, considering that the fish continue to grow throughout their life, although at a slow rate, e.g., in the *Fundulus,* up to 4 years.

Secondly, on Disse's space that you showed in your electron micrographs, is this a consistent finding throughout the several species that you have investigated? The reason I am asking is that in a report in the *American Journal of Anatomy* in 1972, I showed that in the zebra fish and in *Gambusia*, the so-called space of Disse was reduced to the normal intercellular space.

Hinton: We have looked at this after perfusion fixation and after immersion fixation; we come away impressed that the intercellular space in the teleost liver is great, compared with that in mammalian liver. We have also studied different osmolarities of delivery systems in the buffers, and we can induce some changes in that space. We are convinced that it is quite extensive.

Weiss: In the species that I have investigated, considering the osmolarity of the fish tissues in particular, I believed that it could be created, if you were fixing in a hyperosmotic solution.

Hinton: Yes. I am sure you can exert some change on it.

Weiss: The same result occurs in the guppy with dichlorodiphenyltrichloroethane as in your fish treated with polychlorinated biphenyls with their stimulated smooth endoplasmic reticulum. That was reported in 1974, but this does not happen in the zebra fish. I think some fish can respond by developing the smooth reticulum as you demonstrated, but others cannot.

Histological Progression of Hepatic Neoplasia in Rainbow Trout (*Salmo gairdneri*)[1, 2, 3, 4]

Jerry D. Hendricks, Theodore R. Meyers, *and* Dennis W. Shelton[5, 6]

ABSTRACT—The histological progression of hepatic neoplasia has not been as systematically studied in rainbow trout as it has been in rodents. Two putative preneoplastic lesions have been identified, the eosinophilic focus and the basophilic focus, but whether these correspond to similar lesions in rodent livers is not known. Preneoplastic liver lesions in rodents have been extensively characterized histochemically, but adaptation of these techniques to trout livers has not always been successful. Eosinophilic foci consist of hypertrophied cells, enlarged atypical nuclei, and dense glycogen-free cytoplasm. Mitotic figures are also occasionally seen. Usually, these foci have been infiltrated and at least partially destroyed by inflammatory cells, largely lymphocytes. In some liver sections, eosinophilic foci are intact and occasionally an eosinophilic–basophilic transformation can be seen. However, most often basophilic foci appear independently, surrounded by normal heptatocytes, with no indication of a prior eosinophilic stage. The cells of basophilic foci are similar to those of carcinomas: intensely basophilic, mitotically active, devoid of glycogen, and grouped into cords several cells in thickness. These nodules may appropriately be referred to as carcinomas in situ, because the only distinguishing characteristic is the size of the lesion. Attempts at differentiation between benign and malignant liver lesions appear arbitrary. We believe the best classification of the neoplastic liver lesion in trout is a hepatocellular carcinoma because the potential for malignant behavior always exists and, with sufficient time, can often be histologically demonstrated. We have also described our experience with the characteristics of other liver lesions associated with hepatocarcinogenesis. — Natl Cancer Inst Monogr 65: 321–336, 1984.

The classification of proliferative liver lesions in rats resulting from exposure to hepatocarcinogens has received much attention from veterinary and human pathologists. Accordingly, the literature contains numerous descriptions of the various sequential lesions encountered during hepatocarcinogenesis. Fundamental differences of opinion as to the nature and significance of various lesions as well as a lack of uniformity in semantics prompted several efforts toward standardization of a classification. The National Cancer Institute first sponsored a workshop in 1974 on the classification of hepatocellular tumors and related lesions that resulted in a highly useful report (*1*). In 1977, a subcommittee, established by the Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, began a series of meetings to establish a standardized classification of rat hepatic tumors. The end result of this extensive effort was the "Histologic Typing of Liver Tumors of the Rat," (*2*) which presently serves as the standard for researchers in this popular area of cancer research.

Unlike the well-defined classification scheme for experimental rat liver tumors, the classification of similarly induced liver tumors in trout is poorly understood and almost entirely lacking. Since the initial report of an epizootic of hepatoma in hatchery-reared rainbow trout (*3*), limited effort has been given to the development of this species or other fish species as cancer models. Consequently, even less effort has been expended in the characterization of the liver tumors themselves. Therefore, it is most encouraging to see the fruition of this Symposium that resulted from the present interest of the National Cancer Institute in fish species as possible model systems for cancer research.

In this paper, we describe our observations and those of others concerning the identification of hepatic tumors and other lesions resulting from exposure of trout to various hepatocarcinogens (*4, 5*). We will attempt to compare the lesions observed in trout with those described for rats (*1, 2*), but we do not imply that they are necessarily analogous.

ABBREVIATIONS: AFB_1 = aflatoxin B_1; CPFA = cyclopropenoid fatty acid(s); ppb = parts/billion; ppm = parts/million; H & E = hematoxylin and eosin; PAS = periodic acid-Schiff.

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[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Supported in part by Public Health Service grants ES01926, ES00210, ES00040, and ES00541 from the National Institute of Environmental Health Sciences; contract N01-CP85660 from the Division of Cancer Cause and Prevention, National Cancer Institute; and Cooperative Agreement grant CR809344-010 from the National Cancer Institute/Environmental Protection Agency Collaborative Program, Project No. 3, Effects of Carcinogens, Mutagens, and Teratogens in Non-human Species (Aquatic Animals), which is administered by the Gulf Breeze Environmental Research Laboratory, Gulf Breeze, Florida 32561.

[3] Animals were maintained under the guidelines set forth by the Oregon State University and the "Guide for the Care and Use of Laboratory Animals" by the Institute of Laboratory Animal Resources, National Research Council.

[4] Technical Paper No. 6478, Oregon Agricultural Experiment Station, Oregon State University, Corvallis, Oregon 97331.

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[6] We thank Mrs. L. J. Hunter Gray, Mrs. Duen Poocharoen, Mr. John Casteel, and Mr. Theodore Will for their technical assistance.

GENERAL ASPECTS OF TROUT LIVER TUMORS

Spontaneous hepatic neoplasms in wild or cultured rainbow trout are extremely rare. When such tumors do appear in cultured trout, it is generally assumed that previous exposure to a carcinogen has occurred.

Carcinogens and Routes of Exposure

Among the number of chemical compounds proved to be hepatocarcinogens in rainbow trout, the foremost was AFB_1 (*6*). The rainbow trout was the first animal in which this now widely studied mycotoxin was shown to be a hepatocarcinogen. Subsequently, the following compounds have also proved to be carcinogenic: aflatoxin G_1 (*7*), aflatoxin M_1 (*8*), aflatoxicol (*9*), aflatoxin Q_1 (*10*), sterigmatocystin (*11*), versicolorin A (*11*), CPFA (*12, 13*), dimethylnitrosamine (*14, 15*), diethylnitrosamine (unpublished results), nitrosopyrrolidine (unpublished results), 2,6-dimethylnitrosomorpholine (unpublished results), nitrosomorpholine (unpublished results), methylazoxymethanol acetate (unpublished results), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (*16*), benzo[*a*]pyrene (*17*), and dichlorodiphenyltrichloroethane (*5, 18*). The list undoubtedly could be much longer if more compounds were tested. Interestingly, the same types of liver tumors are produced by the action of each of these compounds, some of which vary greatly in their chemical structures. Whether this observation suggests the presence of an oncogene(s) involved in the

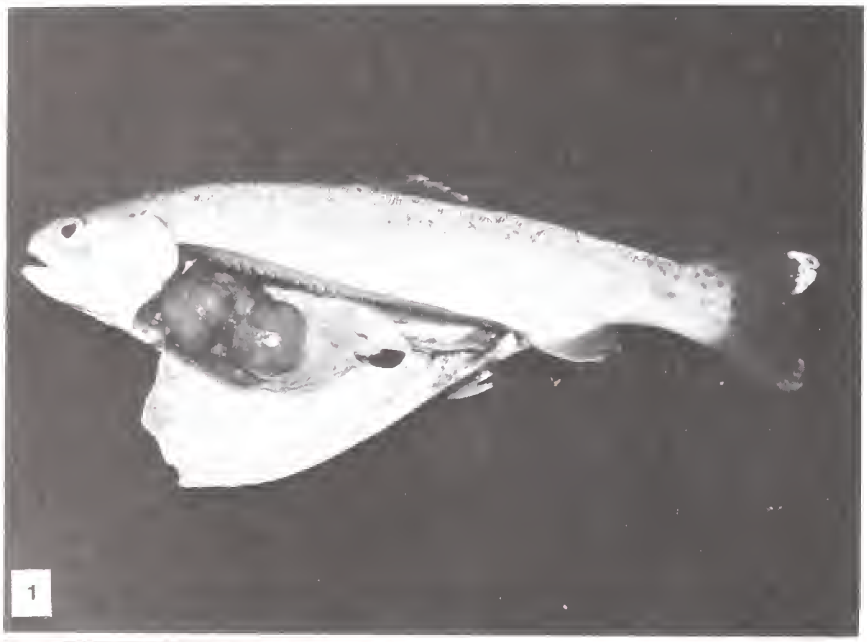


FIGURE 1.—A rainbow trout with 2 large hepatocellular carcinomas resulting from consumption of a diet containing 2 ppb AFB_1 and 50 ppm CPFA for 9 mo.

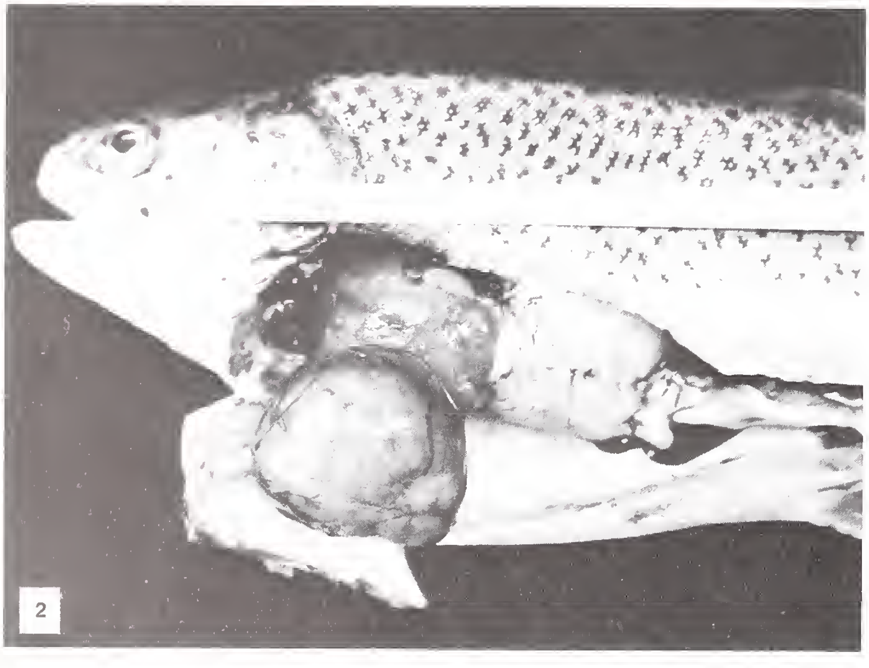


FIGURE 2.—A rainbow trout with a single large hepatocellular carcinoma. This fish had been fed glandless cottonseed meal (containing CPFA) at 25% of the diet for 1 year. Figure is reproduced with permission of the publisher (*13*).

expression of hepatocellular neoplasia in trout is not known, but it is a hypothesis worthy of investigation.

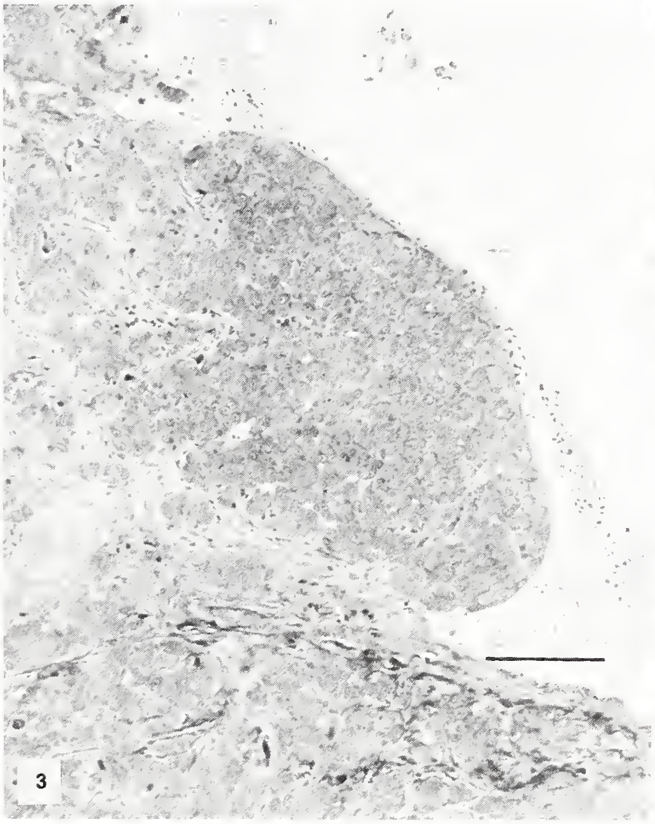
Several routes of exposure have been effective in initiating cancer in rainbow trout including dietary (*4, 5*), ip injection (*19*), and static exposures of embryos or fry to aqueous solutions of carcinogens (*20*).

Gross Features of Trout Tumors

Trout exposed to high doses of strong carcinogens develop in 4–9 months large liver tumors which may be multiple (fig. 1) or single (fig. 2). Low doses of strong carcinogens or high doses of weak carcinogens will usually result in single, small tumors that require 12–18 months to appear. Grossly, small liver tumors are usually pale gray, tan, yellow, or white in color and centrally depressed. The larger tumors may be similar or mixed in color and are more nodular, often containing areas of hemorrhage, necrosis, or fibrosis. The trout liver, variable in shape and inconsistently organized into lobes, does not appear to have a particular region more susceptible to tumor formation. It does seem, however, that tumors appear more frequently on the dorsal convex surface rather than on the ventral concave surface. Generally, most hepatic tumors in trout can be observed grossly on the liver surface, but hand slicing and histological sections are required for one to detect the occasional tumors present internally. Surface tumors are first detectable when about 0.5 mm in diameter. Our usual laboratory procedure is to fix liver tissue in Bouin's solution for routine histology. We observed that fixation in Bouin's fortuitously provided a convenient means of gross tumor detection, particularly for internal growths. Bouin's fixative gives trout hepatic tumors a light yellow color, easily discernible against the darker mottled background of normal trout liver tissue (*21*). Hand slicing fixed livers into 1-mm sections with a razor blade thus permits fairly accurate detection of hepatic tumors as small as 0.5 mm in diameter. Foci of altered cells smaller than 0.5 mm can only be observed histologically in prepared slides.

Metastasis

Metastasis of trout liver tumors occurs infrequently under most experimental conditions. Documented cases of metastasis are available in the literature (*22, 23*) and have been observed in our laboratory. These cases usually involve fish that are 3–6 years old. For instance, Ashley and Halver (*22*) reported that 30% of a group of 4-year-old, tumor-bearing rainbow trout had metastases to other organs. We often observe vascular invasion by neoplastic cells (fig. 3) or tumor emboli, or both (fig. 4) in 12- to



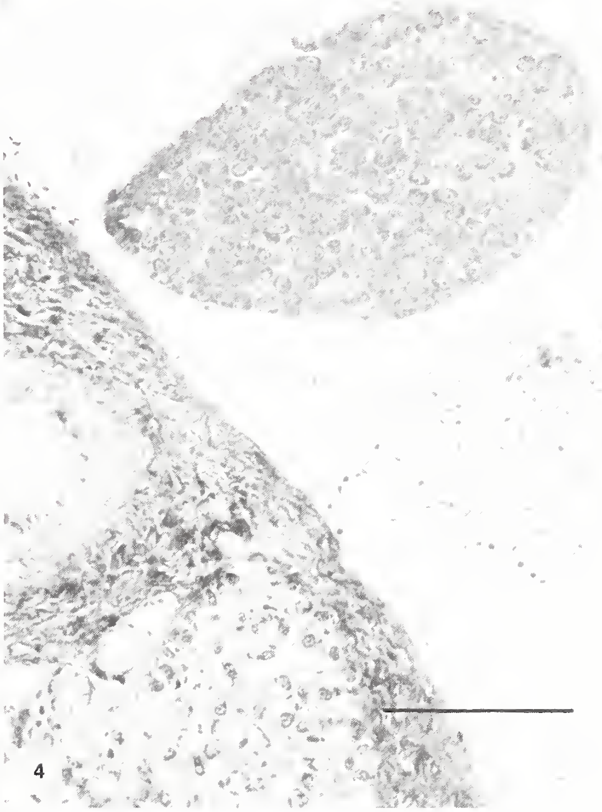


FIGURE 3.—Invasion of a large hepatic vein by a well-differentiated hepatocellular carcinoma in a rainbow trout fed 20 ppb AFB_1 for 4 wk and killed 1 yr later. *Note* the protrusion of tumor tissue through the vessel wall into the lumen of the vein. H & E. Bar = 50 μm. × 320

FIGURE 4.—A tumor embolus in a large hepatic vein from a trout liver having several well-differentiated hepatocellular carcinomas. The vein wall, a small hepatic artery, and normal liver tissue are present in the *lower left* of the photograph. The rainbow trout had been fed a diet containing 20 ppb AFB_1 for 4 wk and sampled 1 yr later. H & E. Bar = 50 μm. × 512

18-month-old hepatic tumor-bearing rainbow trout that have been killed, which suggests the potential for metastases if the fish were allowed to live longer. It is not clear why hepatocellular carcinoma is slow to metastasize in the trout. The low temperatures at which these animals live delay overall cellular metabolism and possibly liver tumor growth, whereas the well-differentiated nature of most trout hepatic tumors suggests weakly malignant behavior rather than the invasiveness found in more anaplastic tumors. However, among the occasional cases of liver tumor metastasis in trout, most have involved well-differentiated tumors of apparent low malignancy.

Transplantation

To our knowledge, only one investigator attempted to demonstrate the transplantability of trout liver tumors. The obvious reason for this is that no inbred strains of rainbow trout have been available in which these experiments could have been performed. Halver (*18*), using a heterogeneous population, attempted to minimize the heterogeneity by using type sera and cross-matching as closely as possible donors with recipients. Using this technique, small fragments of tumor tissue were transplanted to the liver, stomach, spleen, pyloric cecal area, testes, ovaries, cardiac chamber, eye, muscle, and subcutaneous fat of recipient fish. After 90 days, transplanted tumor tissue was found growing in about 10% of the recipients. The tissues in which most transplants survived were the liver, musculature of the stomach, and the pyloric cecal area. Only one successful transplant occurred in a recipient fish which had not been serologically matched with its donor. These results indicated that trout liver tumors are transplantable, and, when syngeneic populations or possibly clones of rainbow trout are available, this characteristic and additional criterion for malignancy should be routinely demonstrable.

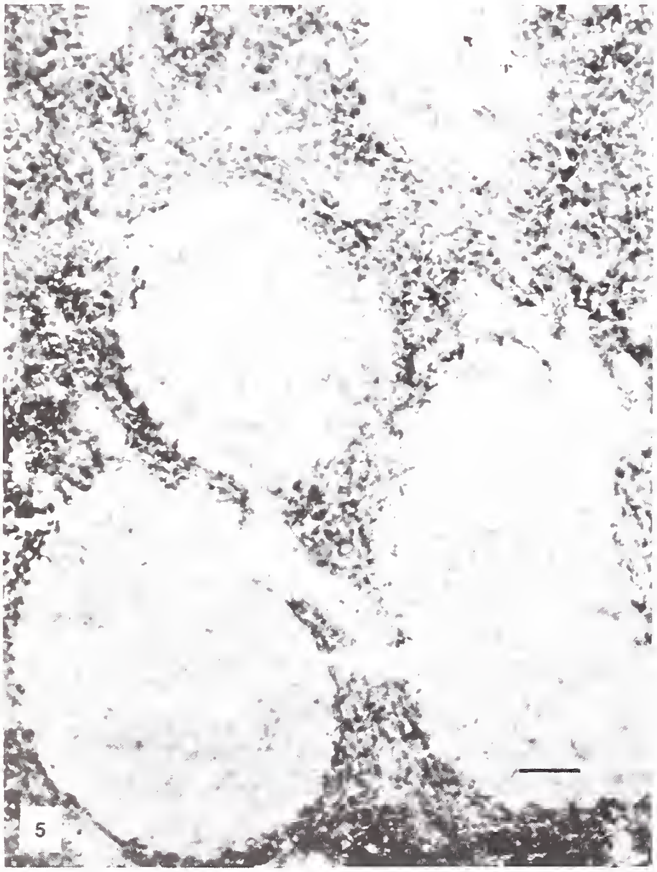


FIGURE 5.—A section of trout liver stained histochemically for glucose-6-phosphatase activity. Four small, probable hepatocellular carcinomas are present and show a marked deficiency of this enzyme compared with that in surrounding normal tissue. Bar= 100 μm. × 80

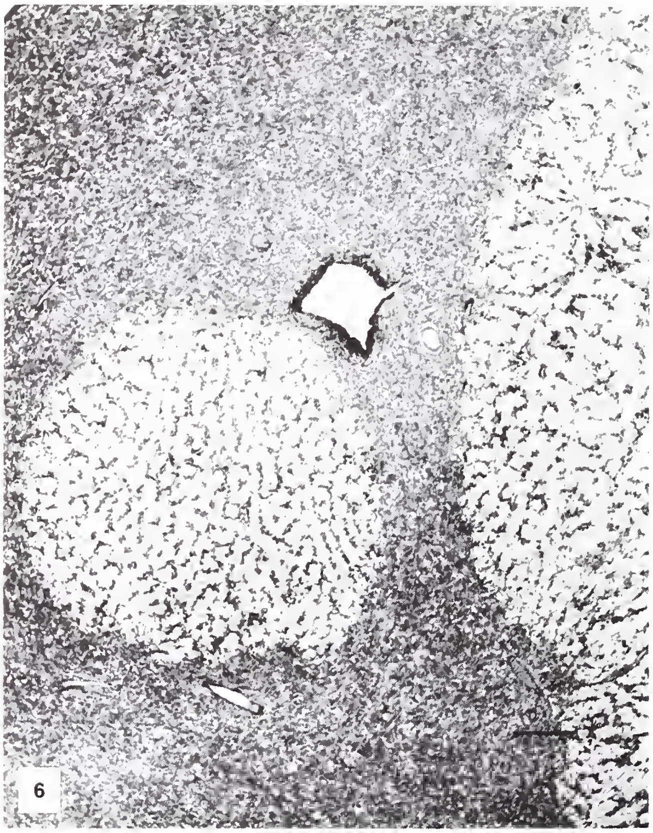


FIGURE 6.—A small glycogen storage nodule and a portion of a larger one in the liver of a rainbow trout fed 405 ppm methyl sterculate (a CPFA) for 11 mo and sampled 2 mo later. Hepatocyte nuclei are normal, but the cytoplasm is engorged with glycogen. Slight compression of surrounding tissue is evident. H & E. Bar= 100 μm. × 80

FOCI AND AREAS OF CELLULAR ALTERATIONS

In rats, 3 distinctive cellular lesions appear in the livers of animals exposed to hepatocarcinogens: the clear, the acidophilic, and the basophilic lesions (*1, 2*). In addition to their descriptive tinctorial characteristics with H & E staining, they also demonstrate a number of functional anomalies when histochemical techniques are used. Some of these include deficiencies of glucose-6-phosphatase and adenosine triphosphatase, abnormal glycogen storage, increased γ-glutamyl transpeptidase activity, and an inability to store iron under siderotic conditions. These lesion types may occur singly, in twos, or all three may be present in the same liver (*1, 2*). They are considered to be preliminary stages in the development of hepatocellular carcinoma and may also coexist with fully developed liver tumors. If these lesions are smaller than the size of a lobule, they are referred to as foci rather than areas which occupy portions of 2 or more lobules (*2*).

In rainbow trout, lesions similar to those types in rats have been observed but are much less characterized for lack of functional studies. The only histochemical staining that we have successfully attempted on trout liver tumors at this time is with glucose-6-phosphatase using the method of Wachstein and Meisel (*24*). Liver lesions large enough to be hepatocellular carcinomas have consistently been deficient in this enzyme compared with normal tissue (fig. 5), although we have not identified the lesions histologically in serial sections. At present, we have no information on the histochemical characteristics of altered liver foci in trout.

In the descriptions that follow, most of the lesions that we have observed are from trout exposed to AFB_1, although similar lesions occur with other carcinogens. Those lesions which appear unique to certain compounds will be noted. We have arbitrarily designated basophilic lesions less than 0.5 mm as foci and greater than 0.5 mm as carcinomas. Eosinophilic lesions are invariably small and classified as foci. Other lesions that are larger than 0.5 mm but not neoplastic are termed nodules.

Clear Cell Focus

A clear cell focus in rats is an obvious lesion due to the apparent emptiness of cell cytoplasm resulting from excessive glycogen storage. A counterpart for this lesion does not occur in rainbow trout when exposed to most classical liver carcinogens. Glycogen-laden cells, which form large nodules that may become larger but do not appear to be associated with neoplastic development, occur in trout exposed to high dietary levels of CPFA ($\geq$200 ppm) for extended periods (fig. 6). The hepatocytes are normal except for the excessive glycogen storage. These nodules are obviously expansive lesions evidenced by compression of surrounding tissue (fig. 6); however, mitotic figures are almost never seen. The significance of this lesion is not known, but we believe it is not comparable to the clear cell focus of rats.

Acidophilic Cell Foci

Foci of parenchymal cells, characterized by intense eosinophilia, are frequently observed in the livers of trout exposed to various carcinogens (*4, 5, 23, 25, 26*). Hepato-

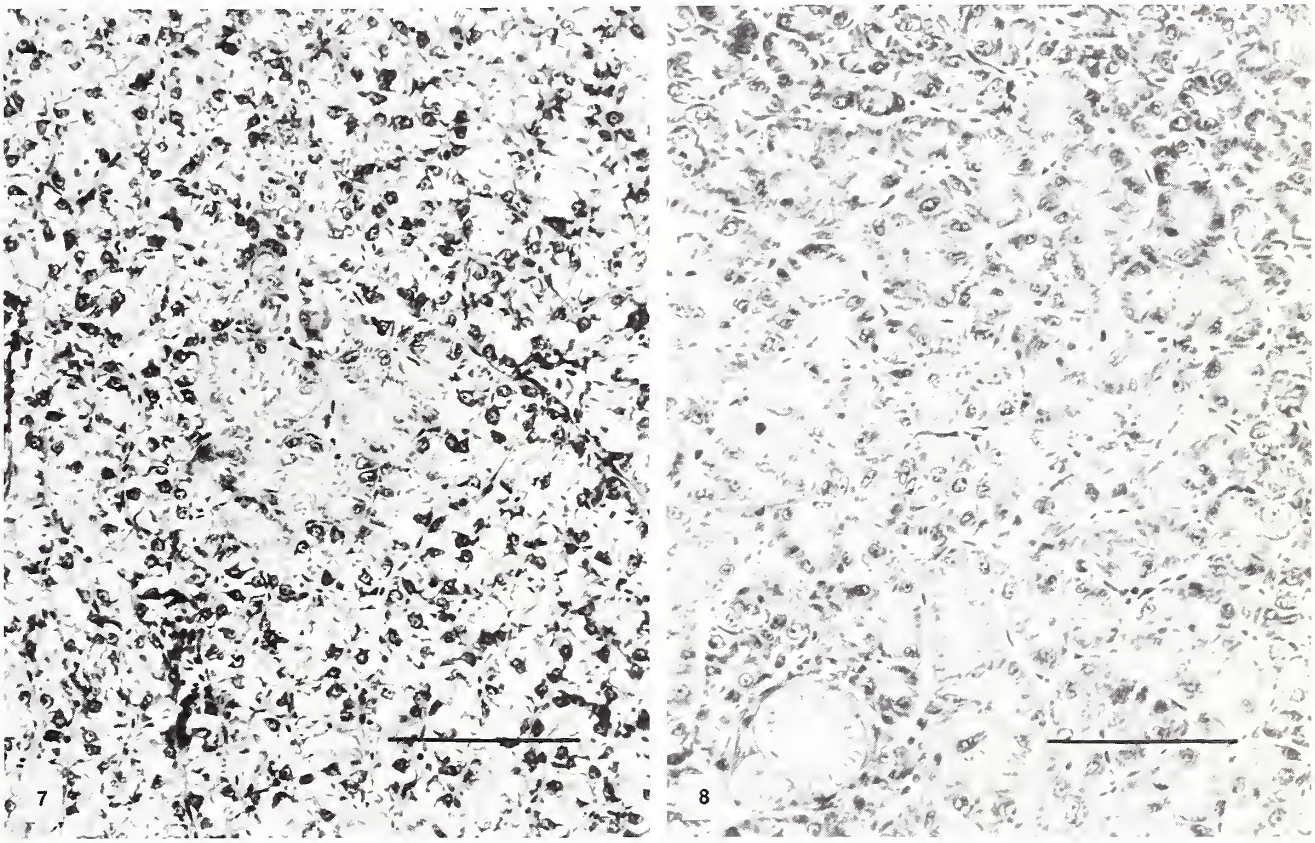


FIGURE 7.—A small focus of eosinophilic hepatocytes in the liver of a rainbow trout fed 20 ppb AFB_1 for 4 wk and sampled 9 mo later. *Note* the hypertrophied cells, enlarged, abnormally shaped nuclei, and lack of glycogen storage compared with surrounding normal tissue. Some cords of eosinophilic cells are continuous with cords of normal cells. H & E. Bar= 50 μm. × 512

FIGURE 8.—A larger focus of eosinophilic hepatocytes similar to those described in figure 7. Normal liver tissue is present *above* and *to the right* of the lesion and around the bile ducts at *lower left*. The trout was fed 20 ppb AFB_1 for 1 mo and sampled 1 yr later. H & E. Bar= 50 μm. × 512

cytes within these foci are hypertrophied and have enlarged often atypical nuclei and dense eosinophilic glycogen-free cytoplasm with a ground-glass appearance (figs. 7, 8). These lesions are generally small (<0.5 mm in diameter) having the characteristic 2-cell-wide hepatic tubulocords which are larger than normal due to individual cell hypertrophy. These cords are continuous with adjacent normal cords and cause no compression. Mitotic figures, though rare, can be observed. Ultrastructural observations of these foci have shown that the intense cytoplasmic eosinophilia is due to an abundance of smooth endoplasmic reticulum (*26*). As mentioned, no data are available on the histochemical properties of these foci, and their significance is uncertain. Nonetheless at least two interesting observations have been made. First and most important is that these foci are often infiltrated and largely destroyed by cells of the immune system, predominantly lymphocytes (figs. 9, 10). Although only speculative, it would appear that these eosinophilic cells are recognized as antigenically foreign by the immune system; thus they are destroyed by cytotoxic lymphocytes. Secondly, because of the above described phenomenon, it is only rarely observed that an eosinophilic–basophilic transformation occurs in trout liver (figs. 11, 12). More commonly, we see the appearance of basophilic foci without any indication of a prior eosinophilic stage.

Basophilic Cell Foci

These lesions are usually small and distinguishable at a size of only a few cells in diameter, but they present a continuum of sizes through large hepatocellular carcinomas (3, 4, 5, 23, *25–32*). They consist of small, unencapsulated, basophilic, glycogen-deficient cells with enlarged nuclei and prominent nucleoli. Small basophilic foci generally have cords 2 cells in thickness but the presence of multicell cords may also be observed. Mitotic figures are often present, but compression of surrounding tissue has not yet occurred (fig. 13). Ultrastructurally, the extreme basophilia is the result of extensive granular endoplasmic reticulum and free ribosomes (*26*). As previously mentioned, these foci usually appear directly in the midst of otherwise normal cells, and only rarely is an eosinophilic–basophilic transformation seen. Unlike eosinophilic foci, basophilic foci rarely elicit an immune response (*33*).

Basophilic foci similar to the focus shown in figure 13 appear as discrete foci, not continuous with the surrounding cords of normal cells. Some basophilic foci (fig. 14) appear less malignant and have cords of basophilic cells that are continuous with normal tissue, fewer or no observable mitoses, and more normal nuclei. The significance of these less malignant foci is not known at this time.

Vacuolated Cell Foci

Bannasch (*34*) described hepatic cells that contained numerous lipid droplets as components of neoplastic nodules in rats. Although we have not observed lipid-containing hepatocytes as a regular component of actual liver neoplasia in trout, we often see foci of lipid-engorged hepatocytes (fig. 15) as a common lesion in liver from trout receiving dietary or embryo exposures of strong carcinogens such as AFB_1, dimethylnitrosamine, diethylnitrosamine, or *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine. These foci of lipid-filled hepatocytes do not appear to be involved in any type of stepwise progression toward neoplasia but represent another toxic effect of these compounds on trout hepatocytes. These lesions usually precede the appearance of visible liver tumors, but they are persistent and later occur with overt hepatic neoplasia. Their presence in a trout liver provides good evidence that hepatocellular carcinomas will eventually develop. They may be more closely related to the lesion of fatty change described by Stewart et al. (*2*).

PRIMARY NEOPLASMS

Our understanding of the neoplastic process in trout, as in rats (*34*), is that the appearance of the basophilic cell type, described above for basophilic foci, signifies that neoplastic transformation is complete. We distinguish between basophilic foci and hepatocellular carcinoma only on the basis of lesion size and compression of surrounding tissue. Because the same glycogen-poor basophilic cells comprise both lesions, we realize this distinction is arbitrary. We might more appropriately call these basophilic foci microcarcinomas or carcinomas in situ, inasmuch as we have evidence that they develop into hepatocellular carcinomas.

No lesion in trout is comparable with the neoplastic nodule of rats that is proliferative and may be composed of a combination of clear, eosinophilic, basophilic, or vacuolated cell types (*2, 34*). It is considered neoplastic, as its name implies, and is an integral part in the rat hepatocarcinogenesis process. Because the most common pathway to neoplasia in trout is directly through the basophilic cell type present in basophilic foci and hepatocellular carcinomas, the designation of a stage, within that continuum of lesions, as a neoplastic nodule, is unwarranted.

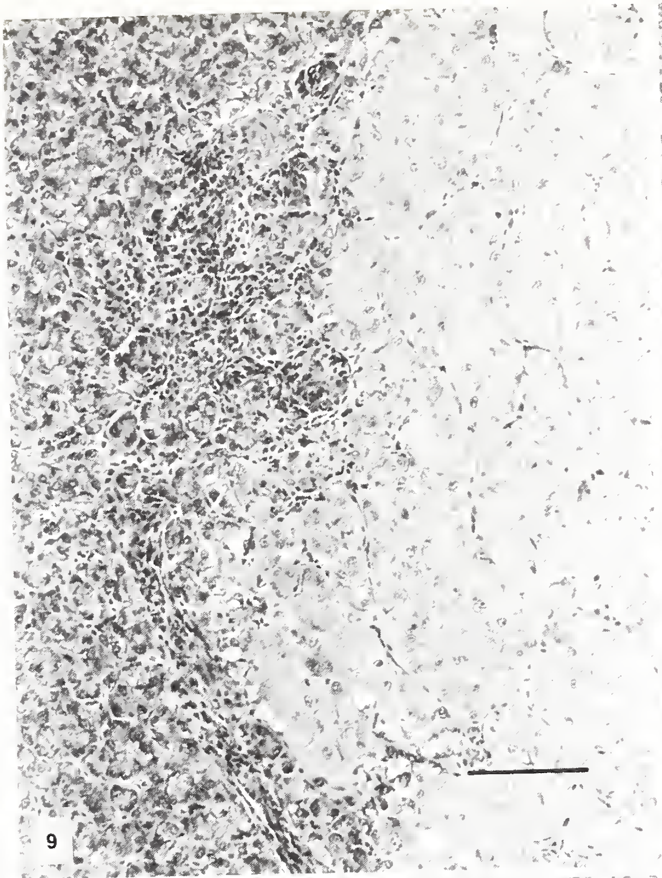
→

FIGURE 9.—A larger focus of eosinophilic hepatocytes with extreme cellular hypertrophy, large, irregular nuclei with multiple nucleoli, and beginning invasion of lymphocytes along the *left* edge of lesion. Carcinogen exposure was dietary AFB_1 at 20 ppb for 4 wk. H & E. Bar = 50 μm. × 320

FIGURE 10.—A more advanced stage of lymphocytic invasion and destruction of an eosinophilic focus in the liver of a rainbow trout fed AFB_1 at 20 ppb for 4 wk. Only a few sequestered groups of eosinophilic cells remain (*arrows*). H & E. Bar = 50 μm. × 320

FIGURE 11.—A neoplastic lesion in the liver of a rainbow trout fed AFB_1. At the top of the photo are cords of normal hepatocytes. In the *middle* and *on the right* are cells of the eosinophilic type that merge directly with deeply basophilic cells at *lower left*. This is a rare observation. H & E. Bar = 50 μm. × 320

FIGURE 12.—A portion of an eosinophilic focus from a rainbow trout liver (exposed to AFB_1) within which individual cells are undergoing basophilic change (*arrows*). This observation is also rarely seen. H & E. Bar = 50 μm. × 512



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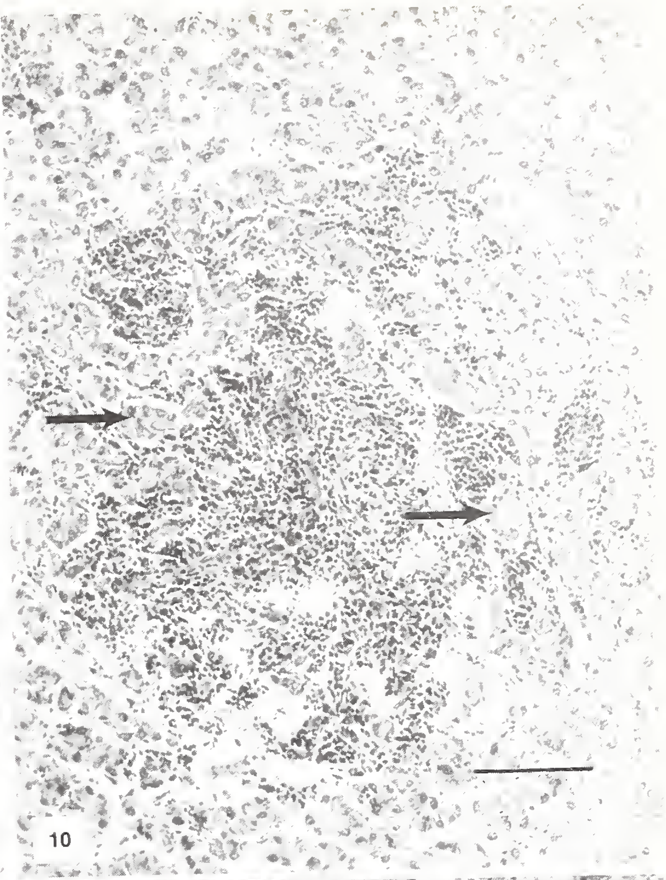

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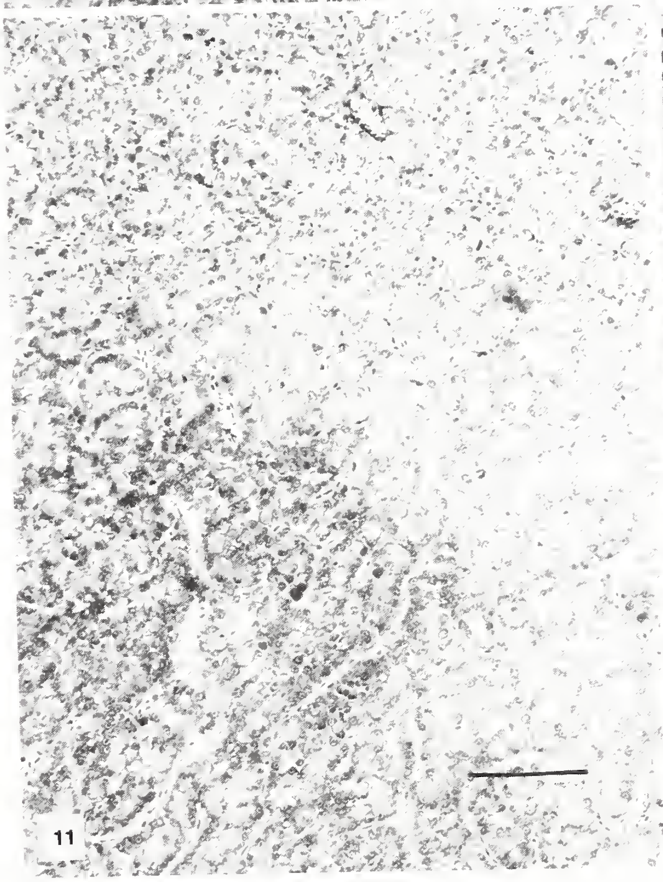

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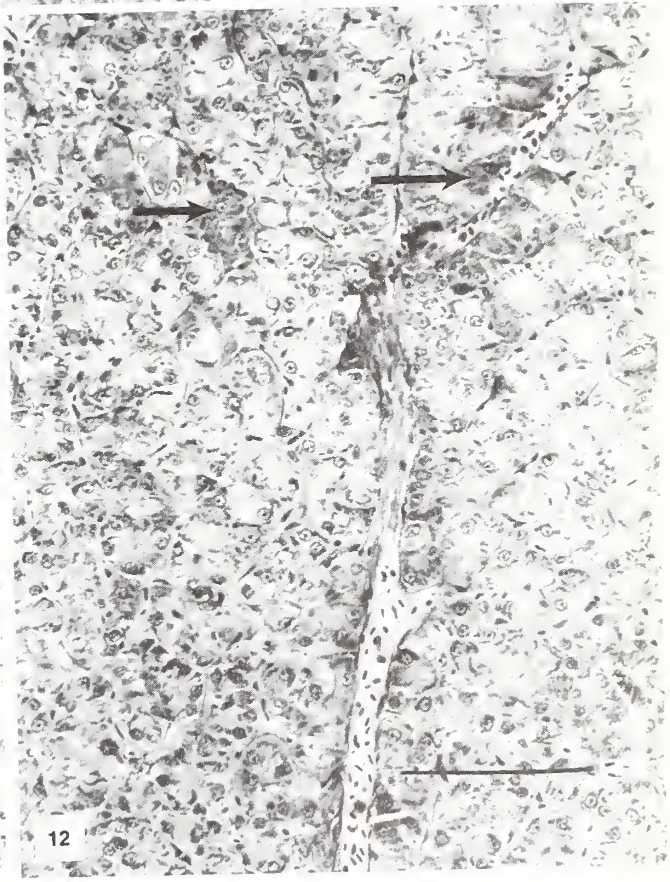

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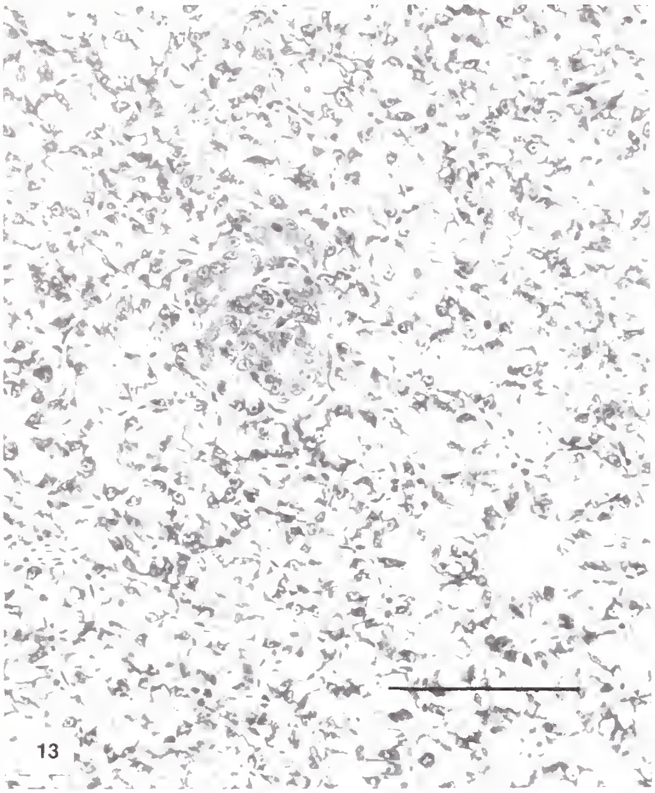
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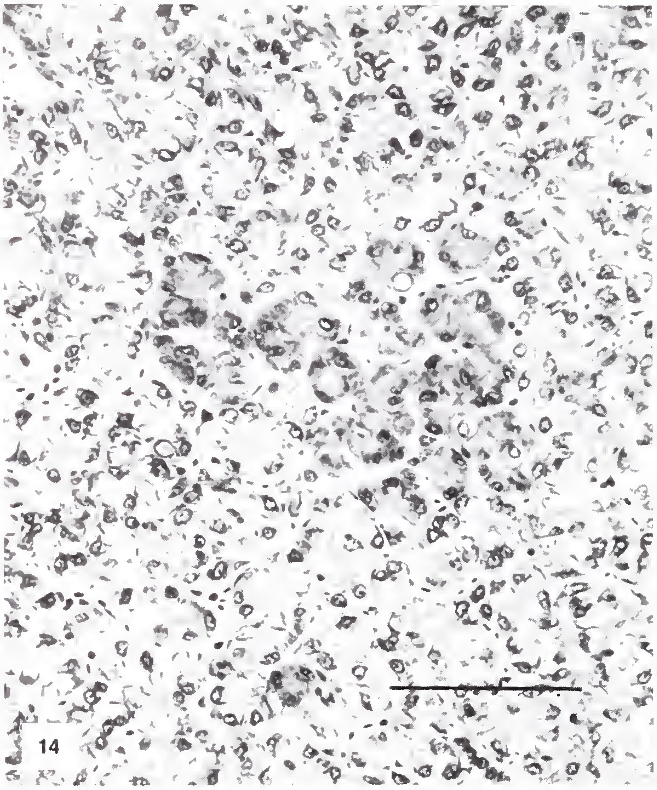
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FIGURE 13.—A focus of basophilic cells in the midst of normal appearing hepatocytes with no indication of a prior eosinophilic stage. The trout was fed 6 ppb AFB_1 continuously for 6 mo. *Note* the small deeply basophilic, glycogen-poor cells, large nuclei, and mitotic figure. H & E. Bar = 50 μm. × 512. Figure is reproduced with permission of the publisher (*4*).

FIGURE 14.—A focus of basophilic cells that are less intensely basophilic and more normal in appearance. Some cords of these cells are continuous with cords of normal cells. The trout was fed 6 ppb AFB_1 for 6 mo. H & E. Bar = 50 μm. × 512

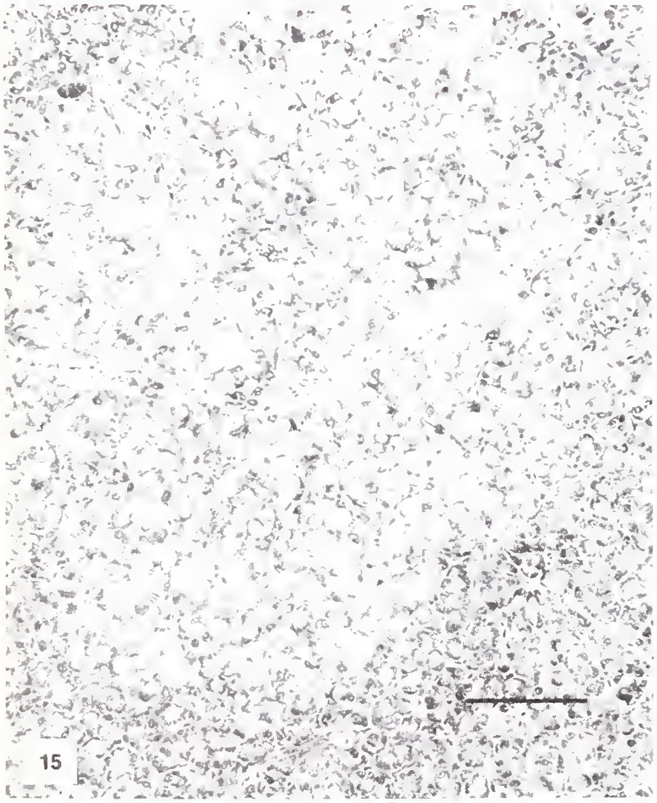
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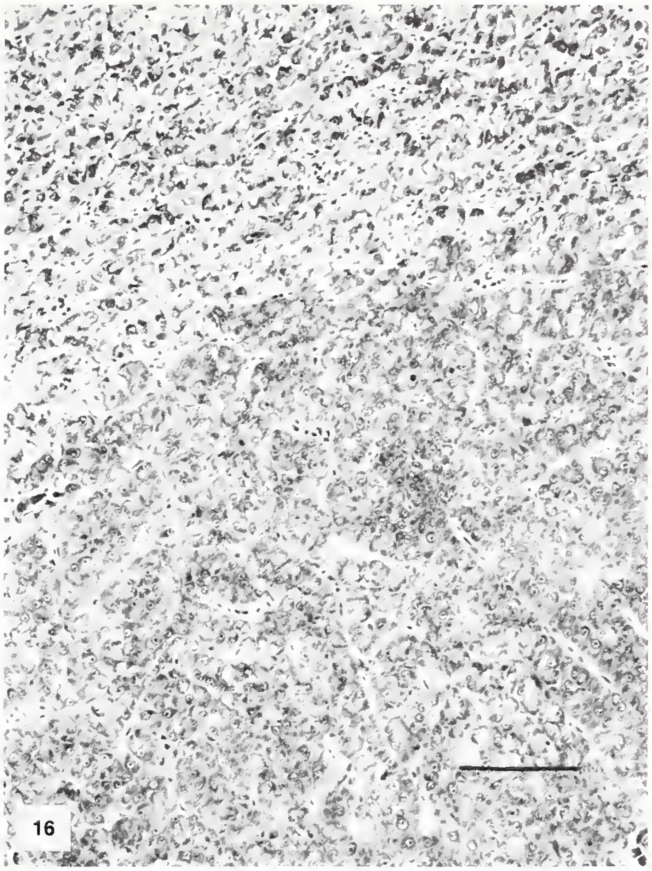
FIGURE 15.—A focus of lipid engorged hepatocytes in the liver of a trout fed AFB_1 at 20 ppb for 4 wk and sampled 12 mo later. Surrounding normal hepatocytes have little or no demonstrable lipid. H & E. Bar = 50 μm. × 320

Trabecular Carcinoma

Trabecular carcinoma is the most frequently observed form of carcinoma in rainbow trout liver. Described by many authors (*3–23, 25–33*), it is composed of broad, multicell cords of basophilic hepatocytes alternating with endothelium-lined sinusoids. The cords of the tumors are discontinuous with those of adjacent liver tissue and lie perpendicular to the compressed cords and sinusoids of the surrounding normal liver tissue (fig. 16). The cells are similar to those described for basophilic foci, but mitotic figures are usually more numerous (fig. 17).

As a variant of this general trabecular pattern, tumors are often observed that have peripheral trabecular components and centrally located hyperplastic bile ducts and supporting fibrous connective tissue stroma (fig. 18). We do not believe the bile duct component is always neoplastic because the bile ducts are mostly normal in structures with few mitotic figures. The low columnar to cuboidal duct cells stain lightly with eosin and have round to oval nuclei. The eosinophilic staining is in marked contrast to the basophilic hepatocellular tumor cells (fig. 19). We have



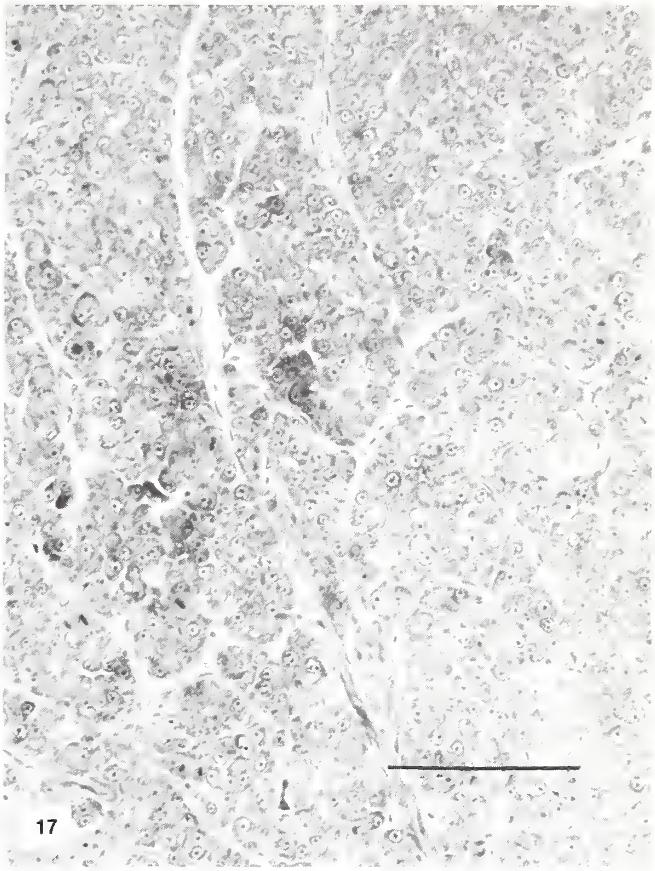


FIGURE 16.—A well-differentiated trabecular hepatocellular carcinoma (*below*) from a trout exposed to 0.5 ppm aqueous AFB_1 as an embryo and killed 12 mo later. *Note* the broad cords of basophilic tumor cells, large vesiculated nuclei with prominent nucleoli, compression of adjacent normal tissue (*above*), and mitotic figures. H & E. Bar=50 μm. × 320. Figure is reproduced from (*11*).

FIGURE 17.—A portion of a well-differentiated, trabecular hepatocellular carcinoma illustrating cellular detail and frequent mitotic figures. Trout was exposed to 20 ppb dietary AFB_1 and killed 12 mo later. H & E. Bar=50 μm. × 512

demonstrated that normal bile ducts in trout are commonly PAS positive in the apical portions of the biliary epithelium (fig. 20). Such staining is also present in many of the hyperplastic ducts within these tumors, which indicates they are of true bile duct origin (fig. 21). Bile duct hyperplasia probably results when an expanding trabecular carcinoma envelops a portal region with its bile duct(s). Among the classical causes of hyperplasia (nutritional deficiencies, endocrine imbalance, irritation), chronic irritation resulting from the mechanical growth of tumor cells or perhaps from a product of tumor cell metabolism (*33*) is most likely the stimulus for proliferation of bile ducts and supportive connective tissue. Sometimes, the bile duct and connective tissue components actually appear to overgrow and replace the hepatocellular component of a tumor (*33*) as is illustrated in figure 22.

Adenocarcinoma

Unlike the tumors having the benign bile ductular hyperplasia described above, other liver tumors of trout have a glandular or acinar component which appears to be neoplastic (fig. 23). This is in addition to the neoplastic hepatocellular elements already present. These glandular areas are composed of duct-like structures with irregularly shaped lumina, some having papilliferous projections and adjacent solid nests of the same cell type. These neoplastic cells have a high mitotic index and are basophilic (fig. 23). The question arises as to whether these neoplastic cells are of hepatocyte or bile duct origin. In rats, researchers disagree on whether biliary epithelium gives rise to adenomatous tumors (*2*). Until this controversy is resolved, it is suggested that these tumors be classified as adenocarcinomas (*2*). Ashley and Halver (*14*) argued that the glandular component was derived from hepatocytes rather than bile ducts and called the tumors adenocarcinomas. At least two histological features of the glandular structures suggest to us that they are not of hepatocyte origin. First there is the presence of connective tissue that invariably surrounds the glands (ducts); in trout liver, this is a normal feature of bile ducts but not of hepatocytes. Secondly, although the staining of the neoplastic liver cells and the glandular cells appears more similar to figure 23 than it does in figure 19, the nuclei are distinctly different from those of neoplastic liver cells. They are more irregular in shape and lack the prominent nucleolus of neoplastic hepatocytes. At this time, we believe that the ductlike structures more nearly resemble neoplastic bile ducts rather than glands derived from hepatocytes. Thus a classification of mixed carcinoma or hepatocholangiocarcinoma would appear appropriate in these uncommon instances. More research on the nature of these structures is obviously needed.

Cholangioma

Occasionally, we have noted proliferative lesions composed entirely of ductular elements and supportive stroma especially in feeding trials with dichlorodiphenyltrichloroethane (figs. 24, 25). The duct cells are columnar in shape, stain lightly with eosin, and have round-to-oval nuclei that stain lightly with hematoxylin; the ducts are often multilocular and mitotic figures are common. These are clearly bile duct tumors with no accompanying hepatocellular component.

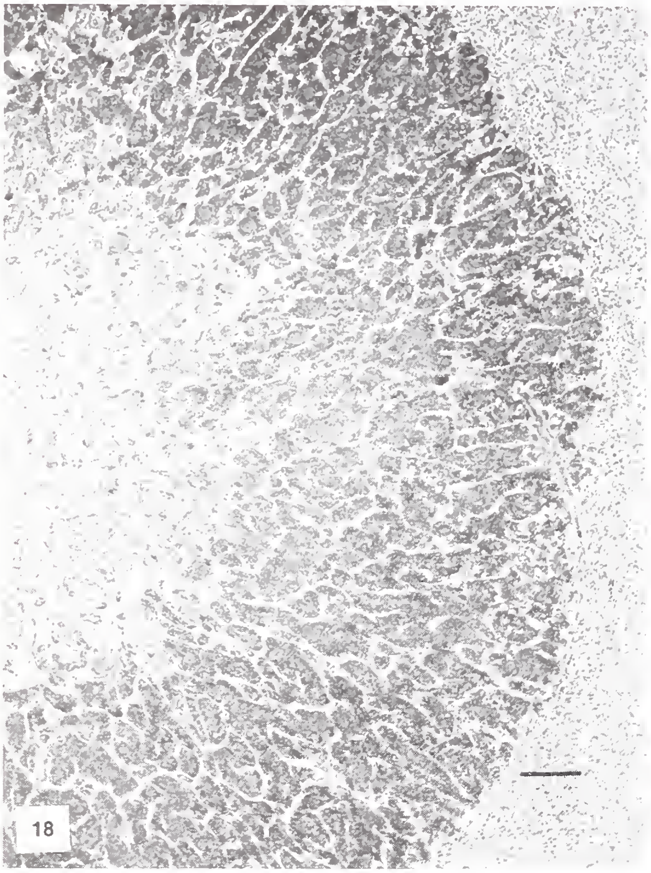
Poorly Differentiated Carcinomas

The degree of anaplasia in trout liver tumors is usually quite restricted, although some tumors do vary from the usual well-differentiated type with its cells of uniform size, shape, and staining characteristics (fig. 26). The more anaplastic tumors have cells that are irregular in size and shape, not organized into well-defined trabeculae, mitotically active, and that may have spindle-like cells. Giant multinucleated cells are rare (fig. 26). Although most trout liver tumors are benign in appearance, the majority of these tumors eventually exhibit malignant behavior. Thus we feel it is speculative to distinguish between malignant and benign liver tumors only on the basis of histological detail. As in rats (*1, 2*), mature benign neoplasms of hepatic origin in trout cannot be determined with certainty.

ASSOCIATED LESIONS

Ceroid Deposits

Ceroid, a polymerized lipid pigment insoluble in organic solvents is both acid-fast and PAS positive. During hepatocarcinogenesis in trout, variable amounts of this pigment are observed in macrophages within melanomacrophage centers surrounding portal tracts of the trout liver (fig. 27).



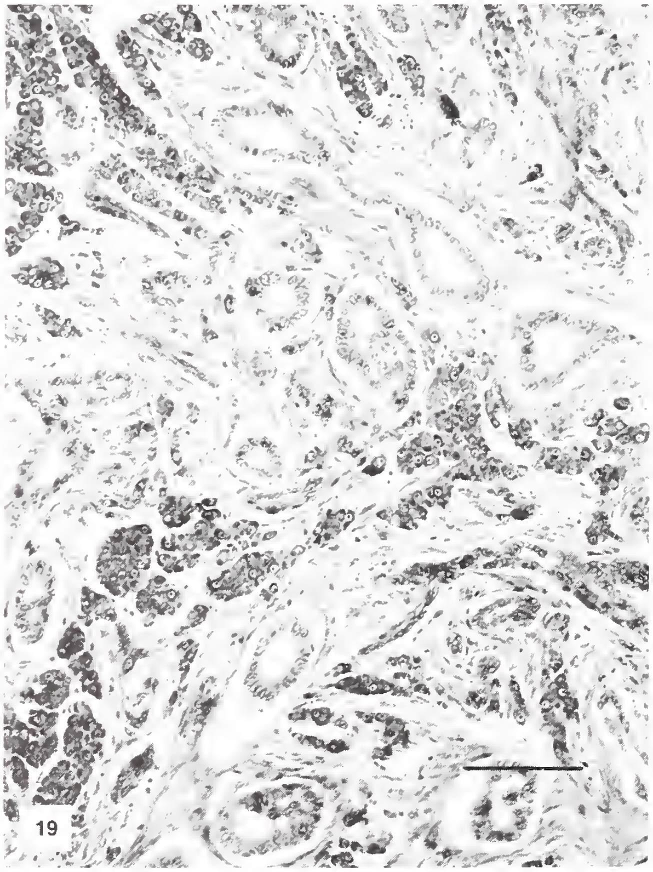
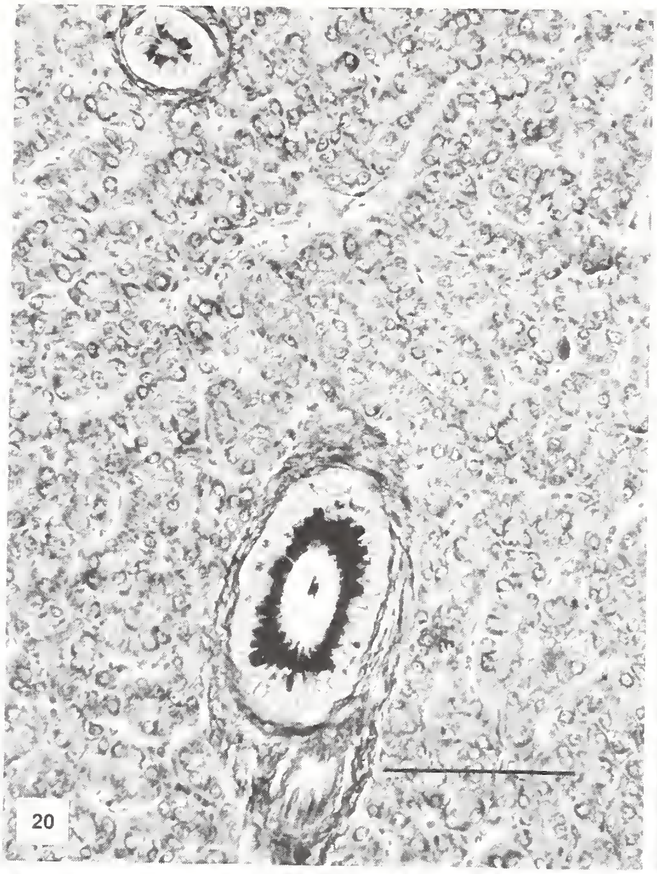
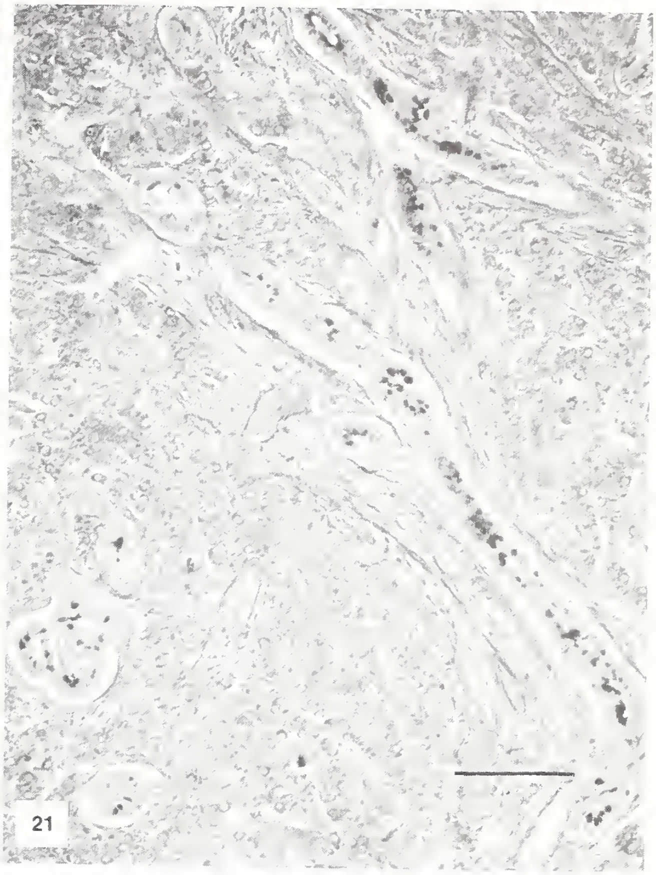


FIGURE 18.—A hepatocellular carcinoma with peripheral basophilic trabecular components and centrally located acidophilic bile ducts and fibrous connective tissue. The trout was exposed to 0.5 ppm sterigmatocystin for 1 hr as an embryo and killed 12 mo later. H & E. Bar = 100 μm. × 80. Figure is reproduced from (*11*).

FIGURE 19.—Central region of a hepatocellular carcinoma initiated by embryo exposure to AFB_1. Photo illustrates the distinct staining differences between the hepatocellular and bile duct components. Trabecular tumor tissue has been sequestered into small islands by the bile ducts and connective tissue. H & E. Bar = 100 μm. × 320. Figure is reproduced from (*11*).





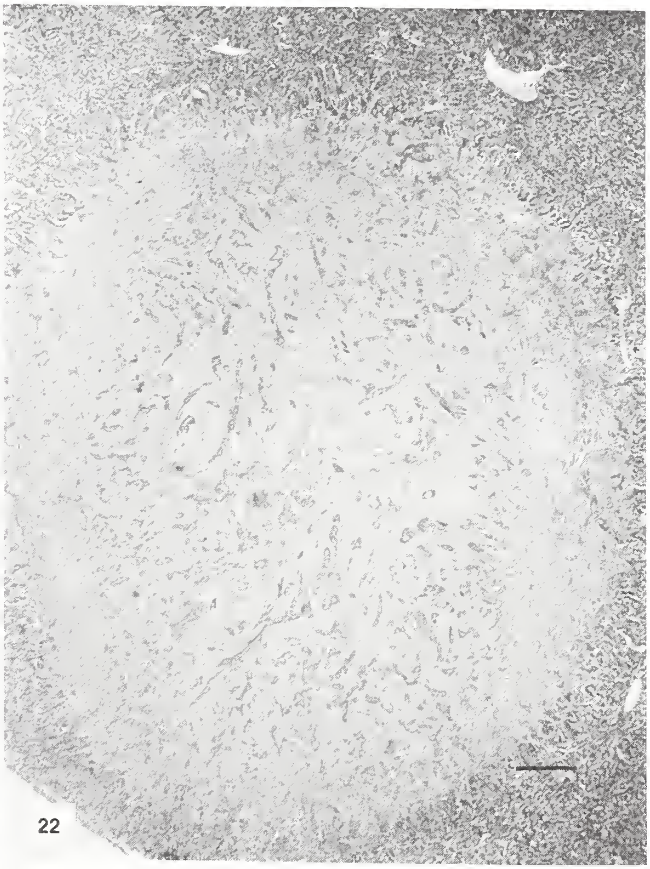


FIGURE 20.—A control trout liver containing a large and small bile duct. *Note* the intense PAS positive staining at the luminal border of the large bile duct cells and the weaker, sporadic staining of the cells in the smaller bile duct. PAS. Bar = 50 μm. × 512

FIGURE 21.—Central region of a hepatocellular carcinoma illustrating the PAS-positive nature of the luminal surface of the bile duct cells. Staining intensity is similar to that in the small bile duct of figure 20. PAS. Bar = 50 μm. × 320

FIGURE 22.—A tumor-like lesion composed entirely of connective tissue and some sparsely located bile ducts. Whether this lesion started as a hepatocellular carcinoma is not known, but the bile duct and connective tissue elements are similar to those seen in other tumors such as shown in figure 18. Trout was exposed to 20 ppb dietary AFB_1 and sampled 12 mo later. H & E. Bar = 100 μm. × 80

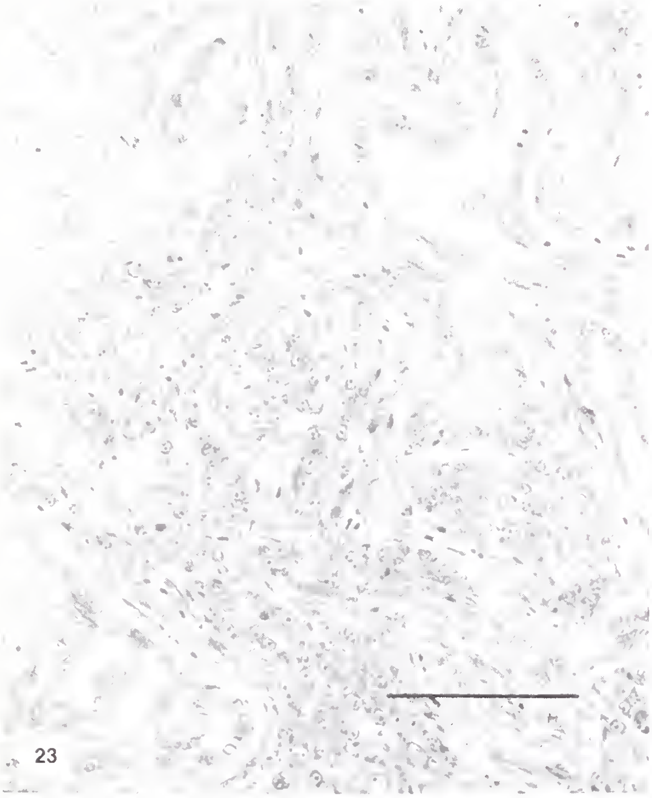
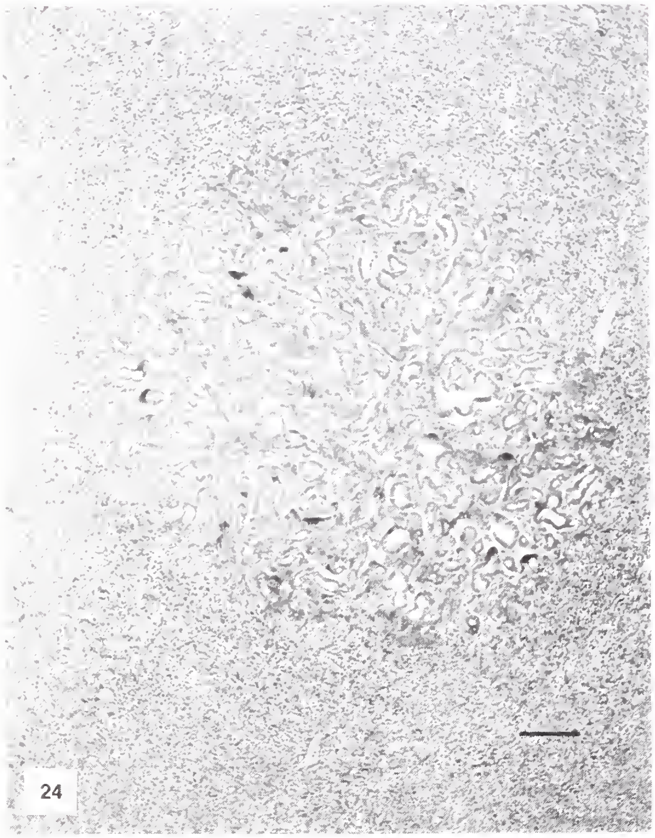


FIGURE 23.—A field from a trout liver carcinoma having both neoplastic liver cells and glandular (ductular) structures. Neoplastic hepatocytes are slightly more basophilic than the glandular cells. *Note* the irregular lumina of the ducts, papilliferous projections, solid nests of cells, mitotic figures, and supportive connective tissue. Trout was fed a diet containing 800 ppm dimethylnitrosamine for 12 mo and killed after 18 mo. H & E. Bar = 50 μm. × 512



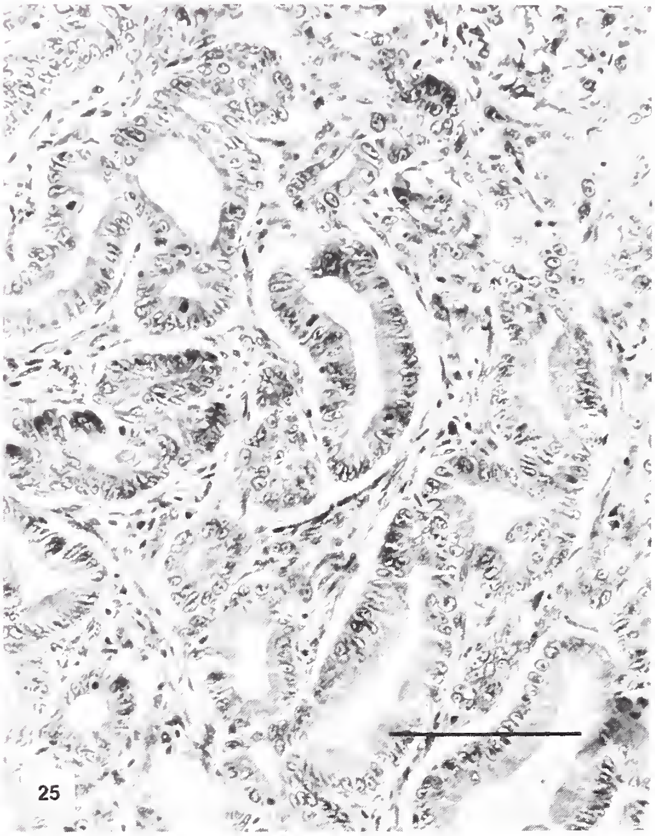


FIGURE 24.—A small cholangioma in the liver of a trout fed 25 ppm dichlorodiphenyltrichloroethane for 18 mo. Lesion is composed entirely of neoplastic bile ducts and supportive connective tissue. H & E. Bar = 100 μm. × 80

FIGURE 25.—Detail of figure 24 showing neoplastic bile ducts. *Note* the multilocular nature of the ducts, frequent mitotic figures, and absence of neoplastic hepatocytes. H & E. Bar = 50 μm. × 512

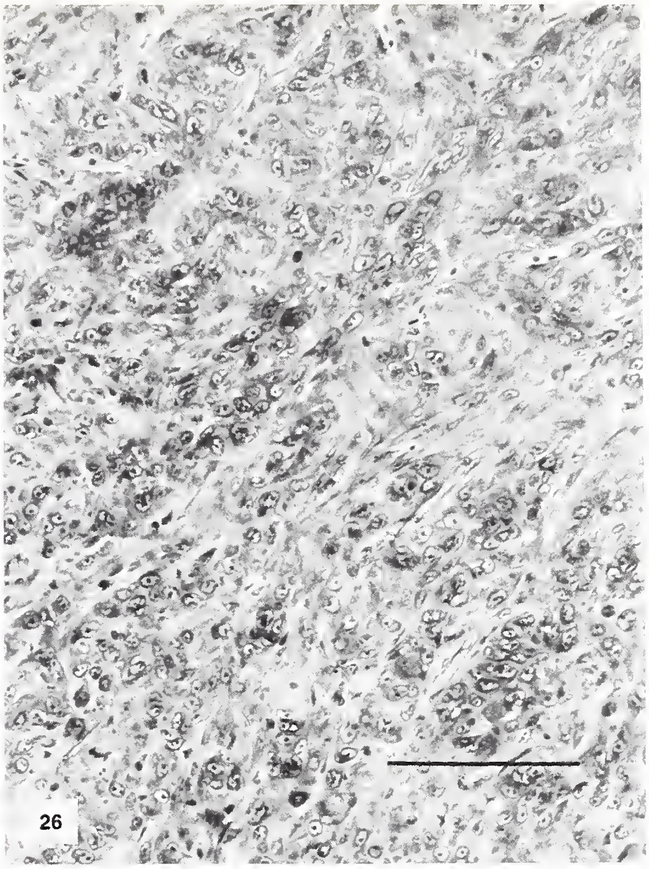


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FIGURE 26.—A portion of a more anaplastic hepatocellular carcinoma from a trout exposed to dietary dimethylnitrosamine. Hepatocytes are irregular in size and shape, not organized into well-defined trabeculae, possible spindle cells are present, and mitoses are common. H & E. Bar=50 μm. × 512

Cysts

Multilocular cysts often occur in the livers of trout treated with hepatocarcinogens. They are often on the surface and are composed of lightly staining cuboidal cells lining empty lumina (fig. 28). Their origin is not clear.

Adenofibrosis

This lesion, occupying large areas of the liver, is composed of immature bile ducts and an abundant connective tissue stroma (fig. 29). Particularly prevalent in trout subjected to prolonged dietary administration of CPFA, it is a rare lesion when trout are exposed to other carcinogens.

Pancreatic Cell Metaplasia

This lesion has been observed in trout fed CPFA or AFB_1. In the CPFA exposure, pancreatic cell metaplasia appeared in otherwise normal liver parenchyma as well as within hepatocellular carcinomas. In AFB_1-treated trout, it has only appeared within the tumor mass of large hepatocellular carcinomas. Special stains have not been applied to

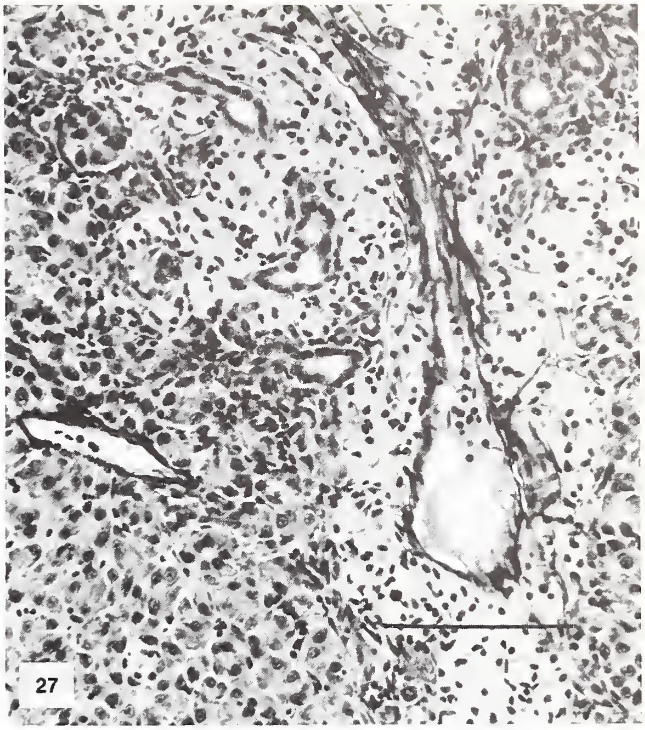

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FIGURE 27.—Ceroid deposits within a melano-macrophage center surrounding a portal area in the liver of a trout fed 50 ppm CPFA for 12 mo. H & E. Bar=50 μm. × 512

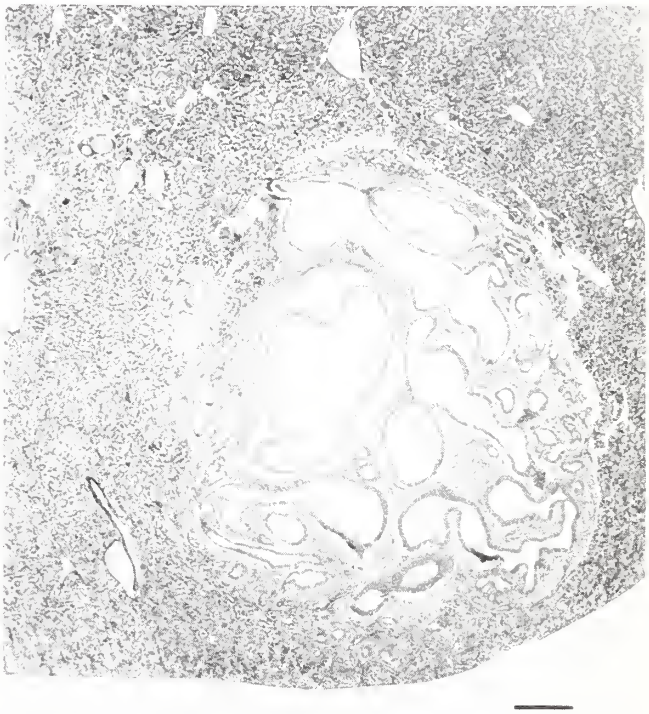

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FIGURE 28.—A multilocular cyst from the liver of a trout exposed to a 0.5 ppm AFB_1 as an embryo. The cuboidal epithelium of the larger cysts is detached from the stroma. H & E. Bar=100 μm. × 80

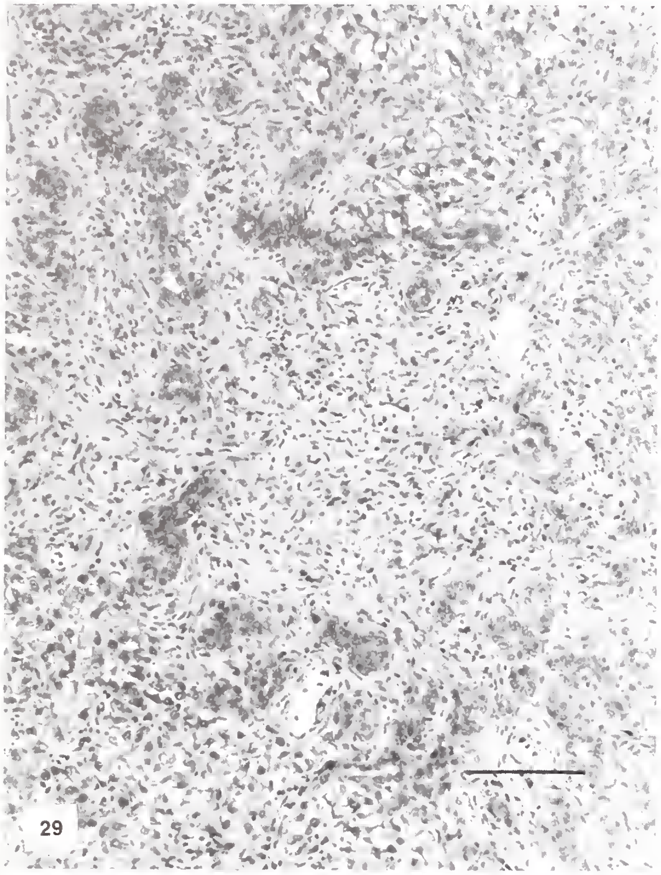

FIGURE 29.—Adenofibrosis in the liver of a trout fed AFB_1-contaminated milk. *Note* the immature bile ductule-like structures, abundant connective tissue, and a moderate number of inflammatory cells. H & E. Bar= 50 μm. × 320

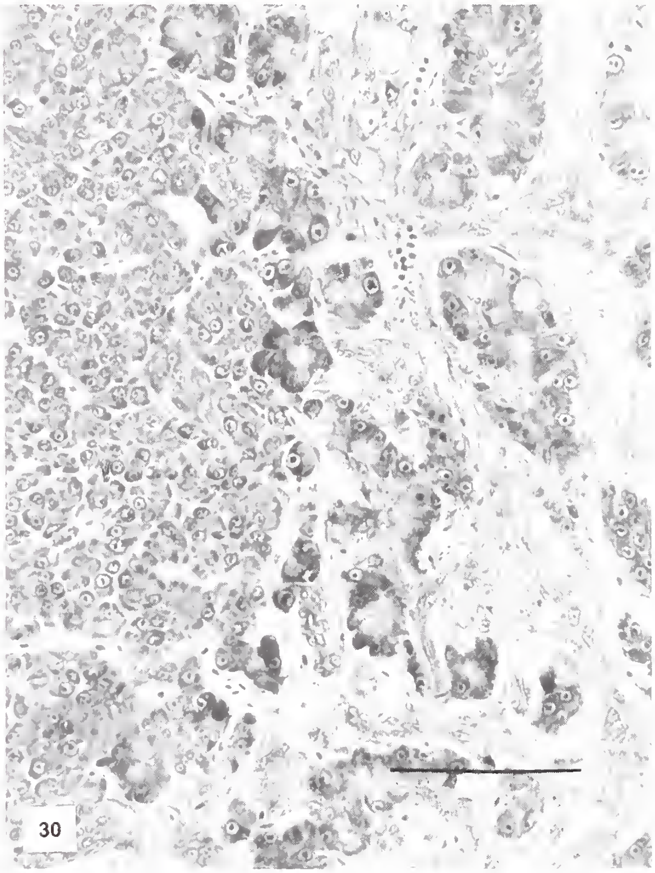

FIGURE 30.—Portion of a hepatocellular carcinoma with both neoplastic hepatocytes (*right*) and pancreatic cell metaplasia (*left*). *Note* the apically located zymogen granules and large nucleoli in many of the pancreatic acinar cells. Fish were fed AFB_1 at 20 ppb for 4 wk. H & E. Bar= 50 μm. × 512

these lesions, but, histologically, the cells are similar to exocrine pancreas, complete with zymogen granules and a large prominent nucleolus (fig. 30).

CONCLUSIONS

This discussion has illustrated the statements made earlier, that although we have made a number of observations concerning cancer of the trout liver, a great deal of effort is still needed for better descriptions and classifications of these neoplastic lesions. From this effort, a better understanding of the neoplastic process may evolve. We hope that these observations will prove useful and encourage others to assist in fitting together the unknown pieces of the puzzle, so that a complete picture of liver neoplasia in trout can be formed.

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DISCUSSION

J. Klaunig: Have you seen any correlation between the number of focal lesions, the basophilic and eosinophilic lesions, that appear early and the number of tumors that are produced? For example, in the rodent livers, there is usually an excess number of focal lesions, and only a few tumors produced.

J. D. Hendricks: That is something we really have not studied, so I cannot give you a good answer. Although I do know that, particularly with feeding trials, with aflatoxin and other carcinogens, we do see a multiple tumor situation, rather than just 1 or 2. Generally speaking, the norm is many tumors per liver, so it indicates that a number of them do progress on to carcinomas.

J. Rice: I have a question for you about the capacity of these lesions to metastasize. Is it not true that in the trout hepatocellular carcinomas metastases may form grossly apparent lesions at sites such as the gills, and if so, how far advanced do the primary lesions have to appear for this to be demonstrable?

Hendricks: Metastases definitely do occur, but generally only late in life. During the duration of most of our experiments, which are usually 1 year in length, we do not see metastases. However, the metastases that have been observed have usually occurred in fish that are 2, 3, or 4 years old; they have been found in various tissues, e.g., gill, the kidney.

Rice: Do you think it is possible that they are found so late in life largely because of a lack of really detailed, systematic histological examinations for micrometastases that might be demonstrable earlier in life?

Hendricks: That is possible because, at least up until just the past few years, most of our histopathology has dealt just with the liver, and so, really, we have not investigated further. I think that is a good possibility.

H. C. Chen: Have you ever seen vascular changes associated with what you saw in the liver tumors or in degenerative changes in the liver, such as peliosis hepatis?

Hendricks: It is not a common occurrence. Another interesting point is that in rodents hemangiosarcomas are fairly common, but I have never seen one in trout liver.

C. J. Dawe: I know that Dr. Hendricks is aware of it but did not mention the fact that the pancreatic metaplasia lesion that he has shown has also been seen in rat livers in experiments in carcinogenesis. I think Kimbrough was probably the first one to describe that. They are similar, and they are a great mystery, at least to me.

We know that the pancreatic rudiments, the anlage for pancreas and that for a liver, both originate close together as diverticulae from the gut. It seems possible, at least, that if these carcinogens are either mutagenic or epigenetic or have epigenetic influences on cells that are transformed, that, rarely, it is possible to get a switchover from liver expression to pancreas expression in some of these lesions. That is merely a rationalistic way of looking at it, and I believe we know nothing about the details.

Hendricks: Recently, Dr. Scarpelli showed the reverse situation in pancreas, a tissue in which he saw differentiation into hepatocytes, so that is just another flip-flop of that idea.

K. Hoover: Recently, investigators at the National Toxicology Program completed testing the compound 2,6-dichloro-*p*-phenylenediamine, and the pathologists there also observed ectopic hepatocytes adjacent to the islets of Langerhans in the pancreases of some treated rats. My questions have been progressively answered, but I would like to know more details about the carcinogenesis of dichlorodiphenyltrichloroethane and benzo[*a*]pyrene in trout.

Hendricks: Dichlorodiphenyltrichloroethane, surprisingly, is quite carcinogenic in the rainbow trout. A dietary level of only 25 ppm induced tumors of the liver in approximately 50% of the animals after 18 months, and it was interesting in that it was definitely sex related. The number of females with tumors was up nearly 75%, and the number of males was down about 20%; it is something that needs to be studied in greater detail.

The report on benzo[*a*]pyrene is some work that we have just recently completed. We have not even looked at all the slides yet, histologically, but we can say for sure that, without any promoting compounds involved, benzo[*a*]pyrene, by dietary exposure, does produce tumors in the rainbow trout.

Hoover: In what concentration?

Hendricks: It was a high one: 1,000 ppm.

J. C. Harshbarger: Some of the tom cod lesions, i.e., those tumors that Mr. Charlie Smith worked with from the Hudson River, are metaplastic pancreatic tissue also. As a matter of fact, quite a lot are. In those tumors that he studied, the hepatocytes were usually grossly hypertrophied. The tumors had large static pools of blood and serum.

Did you ever see the hypertrophied cells and the pools of blood in the trout?

Hendricks: Within the tumor itself?

Harshbarger: Yes.

Hendricks: Not usually. They are pretty uniform as you can see there in the slides.

D. E. Hinton: Have you any ideas about how you might design a study with tumor-laden fish to investigate reversibility of lesions?

Hendricks: Perhaps the best way to answer that is with our embryo exposure, which is a one-time exposure for a brief period and early in life; we can hold these fish for years and those tumors persist. I do not really know what to do beyond that. It is obvious that the tumor continues to develop in the absence of the carcinogen, and it does not regress.

M. Sigel: You may have already broached one of my questions. How does your basophilic cell relate to Dr. Hinton's cell with the increased rough endoplasmic reticulum? Is this the same cell to which he was referring?

Hendricks: Well, certainly, our basophilic cell has an increased rough endoplasmic reticulum. It is really packed with that particular organelle.

Sigel: Would you both agree that this is maybe the essence of a transformed cell in the early stages of carcinogenesis? Thus far, there has been no consensus about anything. Is this the beginning of a consensus perhaps, in two parts of the country?

Hendricks: Yes, I believe that the basophilic cell is the first neoplastic stage that we see. What do you think, Dr. Hinton?

Hinton: Well, I am trying to think. The compound that produced the proliferation, if not carcinogenic, has been shown to be a promoter at least, and it certainly serves as a warning sign.

Sigel: I have a second question, if I may, regarding metastases. Where were the metastases located? Would you care to speculate whether metastases occur when the tumor develops late in life, or whether the fish gets an early tumor, manages to survive for a long time, e.g., for 1 or 2 years, despite a gross lesion in the liver, and then develops metastases? Which is your guess or speculation in this case?

Hendricks: First of all, metastases, like I said before, can be found in a number of different organs. I would say the gill is probably one of the first places; the liver may be a second if it is an embolism-type transfer through the heart, but the first place it is going to lodge is the gill.

I believe that a tumor has to be there a long time before it does metastasize. Generally speaking, our fish are much more sensitive, as young individuals or as fry or embryos, than they are as older adult fish. I think it is a situation in which the tumor has been present for a considerable period before it does metastasize.

J. Stegeman: What was the purity of the dichlorodiphenyltrichloroethane that you used?

Hendricks: It was para para prime form. It was not a pesticide-type formulation.

J. Couch: Going back to the question that Dr. Sigel raised concerning the proliferation of rough endoplasmic reticulum, we have to remember that in pretransformed cells you can have the increase in rough endoplasmic reticulum in a basophilic cell resulting from induction or enhancement of the proliferation of reticulum. In the transformed cell, which may be the authentic neoplastic cell, you may have rough endoplasmic reticulum for different functional reasons. This would not negate what you said because you thought that the transformed cells that were neoplastic cells also had the rough endoplasmic reticulum. I think we have to realize that both the nontransformed and transformed cell may have this characteristic for different functional reasons.

Effects of Temperature and Partial Hepatectomy on the Induction of Liver Tumors in *Oryzias latipes*[1, 2]

Yoriko Kyono-Hamaguchi[3, 4]

ABSTRACT—When the medakas, *Oryzias latipes,* were treated with diethylnitrosamine (DENA) for 6–8 weeks, large liver tumors grew in many of them at 11–13 weeks. In the fish with fully developed tumors, some aneuploid hepatic cells, detected by DNA-Feulgen cytofluorometry, were already found after 3–6 weeks of the DENA treatment. The allogeneic transplantation of liver grafts into eye chambers showed that the grafts obtained from DENA-treated or tumor-bearing fish could survive for longer periods than those from untreated fish. Effects of temperature on liver tumorigenesis were examined at 5–8° C and at 22–25° C. When the fish were treated with DENA at a high temperature, small tumor foci appeared during the treatment, and they grew to large tumors if the fish were kept at a high temperature afterward. Most of the fish had no tumors with the treatment given at a low temperature. Only a few tumors were found in the fish treated with DENA at a low temperature and then kept at a higher one. In the measurement of the labeling and mitotic indexes of liver cells, DNA synthesis and cell divisions occurred during the DENA treatment at a high temperature. A close relationship between cell proliferation and appearance of the histological changes in liver tissue was observed. Three to 4 days after a partial hepatectomy was performed on each medaka, mitotic figures of liver cells were frequently observed, and the maximum mitotic index of more than tenfold the control level was obtained at 4 days. The enhancing effect of the partial hepatectomy on the liver tumorigenesis by DENA treatment was examined. —Natl Cancer Inst Monogr 65: 337–344, 1984.

Several experiments dealing with chemical carcinogenesis in small aquarium fish conducted in recent years (*1*) brought some of the advantages of their use as an experimental species to the attention of investigators. Large numbers of fish can be used in a study with less space and money than mammals require and without the special facilities needed for large fish. Also, large numbers of fish can be bred, raised, and maintained more easily than can mammals. Sato et al. (*2*) and Pliss and Khudoley (*3*) succeeded in the induction of neoplasia in guppy liver. Ishikawa and associates (*4*) reported hepatomas in the medaka treated with DENA and pointed out their high susceptibility to this carcinogen. Aoki and Matsudaira (*5*) also detected hepatic tumors in medaka after treatment with methylazoxymethanol acetate.

We succeeded in inducing liver tumors in the medaka by DENA treatment several years ago and found that the temperatures during and after the DENA treatment had a striking influence on tumor development (*6, 7*). One of the reasons for increased tumor incidence at the high temperature was considered to be related to the high proliferative activity of liver cells. Thus we assessed the proliferative potency of liver cells by measuring the labeling index with [^{3}H]dThd and the mitotic index at different temperatures (*8, 9*). Furthermore, we (*10*) attempted partial hepatectomies in medakas to determine the effect of artificially induced liver cell proliferation on DENA tumorigenesis. In addition to the histological observations, the measurement of nuclear DNA contents of liver cells suggested that a qualitative change of liver cells occurred at a certain stage of tumorigenesis (*8*). Subsequently, we performed allogeneic transplantation of liver grafts treated with DENA to assess any difference between reactions of these grafts and those of the controls.

MATERIALS AND METHODS

Animals.—In this study, the himedaka, an orange-red variety of the medaka, *Oryzias latipes,* was used. Most of the medaka were 1-year-old males. The fish were kept in tap water without aeration and fed on dry food (Tetramin, Tetra Werk, Melle, Federal Republic of Germany) once a day. During the experiment, the glass tanks containing the fish were covered with glass plates with a small opening for air and were illuminated with fluorescent light from 6:00 a.m. to 7:00 p.m. The temperature was controlled at 25 ± 2° C except for the experiment on temperature effect.

Treatment with diethylnitrosamine.—The DENA (Tokyo Kasei Co., Ltd., Tokyo, Japan) was added directly to the water. We adjusted the concentration to 100 ppm by changing the water weekly. Twenty to 30 fish were kept in an 8-liter glass tank. Used DENA-treated water was collected in a polyethylene tank to which HCl was added; it was then neutralized with sodium hydroxide and discarded.

ABBREVIATIONS: DENA = diethylnitrosamine; ppm = parts/million; LI = labeling index(es); MI = mitotic index(es).

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[4] I thank Dr. Nobuo Egami for his guidance and discussions, Dr. Akihiro Shima for his instructions for the measurements of the nuclear DNA contents, and Dr. Kunihiko Kator for his assistance in the presentation of the paper.

After the DENA treatment for 6–8 weeks, the fish were transferred to normal tap water; this water was also changed once a week.

Histological preparation.—The fish were totally immersed and fixed in Bouin's solution, and the liver was dissected, dehydrated in alcohol, and embedded in paraffin according to a routine procedure. Serial sections were made at 6–8 μm except for the study during which measurements of the LI and MI were done. The staining was done with hematoxylin and eosin.

DNA–Feulgen cytofluorometry.—The procedures for DNA–Feulgen cytofluorometry of liver cells were described in detail (*8*). Smear samples were prepared from 2 to 3 fish and processed for nuclear Feulgen staining and autoradiography. The extent of labeling of nuclei within the hepatocytes was ascertained, and then the DNA contents of about 300 nuclei/fish were measured with an automatic digital microfluorometer (Olympus MMSP-RF-S; Olympus Co., Tokyo, Japan).

Transplantation of liver grafts.—Liver grafts were obtained from the fish treated with 100 ppm DENA for 0, 1, 4, and 6 weeks or from the tumor-bearing fish, which were treated with 100 ppm DENA for 6 weeks and then kept in tap water until 13 weeks. The grafts were minced in physiological salt solution for fresh water fish, and the pH was adjusted to 7.3 with sodium bicarbonate (*11*). The recipients (same sex as the donor) were anesthetized with tricaine methane sulfonate (Sankyo Co., Ltd., Tokyo, Japan). While each was under a dissection microscope, we cut the cornea at the upper portion of the eye by a short, quick jab with a sharp knife and slid the liver graft into the anterior eye chamber with fine forceps. The fish were kept in physiological salt solution for the first 3 days after the operation and then were put in tap water. The behavior of the transplants was observed every 4 or 5 days under a dissection microscope for 20–21 days and then each was fixed for histological observation.

Treatment with diethylnitrosamine at different temperatures.—We performed 2 experiments to ascertain the effects of temperature. 1) The temperature during the DENA treatment for 8 weeks was controlled at 25 ± 2° C or 8 ± 2° C and maintained at 25° C afterward (*6*). 2) Four experimental groups were treated with 100 ppm DENA for 8 weeks. The water temperature was controlled at 22 ± 2° C or 5–6° C. After treatment, 1 of the 2 high-temperature groups was maintained at 22 ± 2° C (HH-group), whereas the other was transferred to tanks held at 5–6° C (HL-group). Similarly, 1 of the 2 low-temperature groups was maintained at 5–6° C (LL-group), whereas the other was transferred at 22 ± 2° C (LH-group). In addition, control groups were kept at high or low temperatures throughout the experiment. All the fish were killed at 12 weeks, and histological observations were made (*7*).

Labeling and mitotic indexes of liver cells from fish held at a high temperature.—The fish were treated with 100 ppm DENA for 6 weeks and then transferred to normal tap water. At several points during the experiment, 5–7 DENA-treated fish and 4 controls were given injections of [^{3}H]dThd 2 hours before they were killed. Autoradiography was performed by the routine procedure as described in (*8*). We obtained the LI by counting more than 2,000 hepatic parenchymal cells and 1,000 nonparenchymal cells (other than the parenchymal cells or erythrocytes).

At 1, 2, and 3 weeks of the DENA treatment, another 4 fish were placed in 2×10^{-4} *M* colchicine solution (Wako Pure Chemical Industries, Ltd., Tokyo) for 4 hours and then fixed in Bouin's solution. To obtain the MI, we counted the mitotic figures of more than 1,000 liver cells/fish.

Labeling index of liver cells from fish held at a low temperature.—The fish were treated with 100 ppm DENA for 6 weeks at 7 ± 1° C; then they were transferred into normal tap water and kept at the same temperature (LL-group) or 25 ± 2° C (LH-group). Those fish maintained at a low temperature were acclimated at 25 ± 2° C for 2 hours before they were given injections of [^{3}H]dThd and kept at the same temperature for 2 hours until the fixation, i.e., a total of 4 hours at 25 ± 2° C. The procedures of autoradiography were the same as those described above. More than 1,000 hepatic parenchymal cells were observed/fish, and 6–7 fish were killed at each point.

Partial hepatectomy.—Before the operation, the fish were anesthetized with tricaine methane sulfonate and their body weights were measured. The abdominal side was sterilized with ethanol. Then we made a small incision (with ophthalmological scissors) in the upper portion of abdominal skin close to the septum. A Pasteur pipette connected to a vinyl tube was inserted into each body cavity and almost one-half of the liver was drawn out and cut off with a sharp thin knife. To prevent the extrusion of the intestine, we covered the incision with thin silk cloth soaked in Aron-alpha A (Sankyo Co., Ltd., Tokyo), which is an adhesive used in surgery. The fresh weight of the removed liver was measured. The fish that had the partial hepatectomies were kept in the physiological salt solution containing 20 μg streptomycin sulfate/ml and 20 IU crystalline penicillin G potassium/ml (both from Meiji Seika Co., Ltd., Tokyo). Fish receiving sham operations served as controls. Each surgically treated fish was reared in a glass dish.

On days 1, 2, 3, 4, 5, and 7 after the operation, the fish were treated with 2×10^{-4} *M* colchicine for 4 hours before fixation. We calculated the MI from more than 1,000 liver cells/fish, using 5–12 fish per each point.

Also, the whole livers of other intact fish in the same group with similar body weights were removed and weighed. Then the ratio of the weight of partially hepatectomized liver to that of the whole liver was calculated.

Treatment after partial hepatectomy.—Treatment with DENA after the partial hepatectomy was started 3 days after the operation. We kept 2 fish/glass dish containing 100 ml physiological salt solution or tap water and added DENA at concentrations of 100 or 200 ppm for 4 or 7 days. The dishes were covered and kept at 25–28° C. The DENA solution was changed either every day or every other day. After treatment, the fish were reared in tap water at 25 ± 2° C until they were killed at 6, 11, or 15 weeks of the experiment. We made our histological observations on the serial sections.

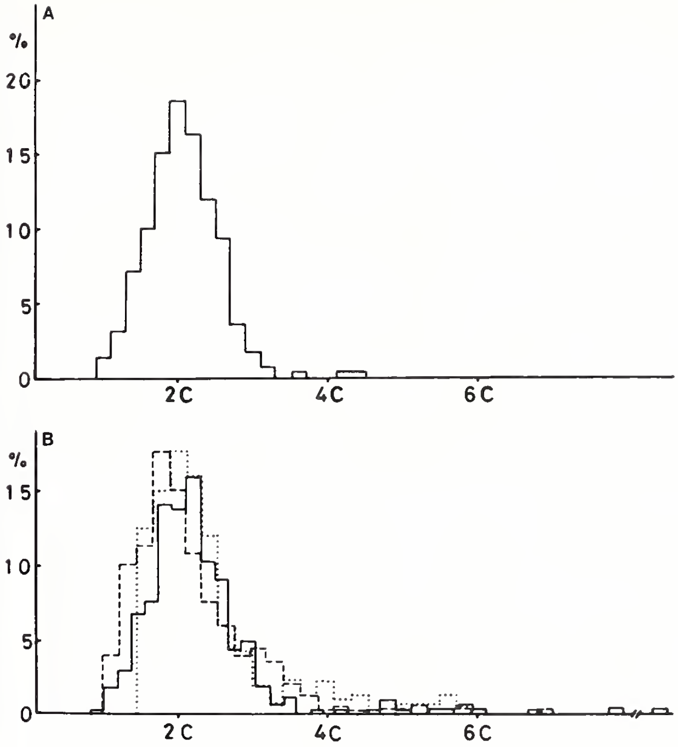

FIGURE 1.—Frequency distribution of nuclear DNA contents in liver cells. A) typical histogram of control fish: B) 3 histograms of tumor-bearing fish obtained at 13 wk of the experiment. More than 300 nuclei were examined/fish. *Abscissa*= DNA content-based ploidy class; *ordinate*= percentage of frequency.

RESULTS

Characteristics of Liver Tumors Induced by Diethylnitrosamine in Medaka

Histological Changes

Because the histopathogenesis of liver tumors in medakas has been reported (*10*), we will only describe the histological changes found in the male medaka treated with 100 ppm DENA.

About 1 week after the fish were treated with DENA, the cytoplasm of hepatic parenchymal cells appeared weakly eosinophilic, whereas that of normal hepatocytes was barely stained. Focal necroses were observed at approximately the same time, or at 1–2 weeks after the treatment. Soon after the appearance of the necroses, multiple basophilic areas appeared and were spread through the livers at 2–3 weeks; basophilic cell foci, in which cells formed clusters, appeared at 4–6 weeks. The cells in the foci contained scanty cytoplasm, and each nucleus had a single prominent nucleolus. The features of these cells were similar to those of the cells in large tumor nodules. After the appearance of small foci, they seemed to grow rapidly and tumor nodules of various sizes developed at 11–13 weeks. The common tumors were considered to be trabecular hepatomas or liver cell carcinomas.

Nuclear DNA Content of Liver Cells

At 13 weeks, approximately 5% or more of the liver cells in tumor-bearing fish had abnormal nuclear DNA content exceeding 4C (fig. 1). Such cells had never been observed in the normal livers of medakas (*12*). According to our (*8*) measurements of DNA throughout the course of liver tumorigenesis, we found that DNA content was increased in cells at 3 weeks after DENA treatment was initiated, and the levels continued afterward.

Transplantation of the Liver Grafts

More than 75% of the transplants were successful, i.e., the grafts did not drop out of the eye chambers nor did the surgery cause the death of the recipients. The transplanted liver grafts scarcely grew in the anterior eye chambers even if the grafts were obtained from fish treated with the carcinogen or the tumor-bearing fish. No grafts degenerated completely during our observations. Small pieces were observed in most recipients even at 20–21 days. However, during the first 12–13 days, the process of degeneration between the grafts obtained from the controls and those from the DENA-treated or tumor-bearing fish differed (table 1). All the grafts from the controls decreased in size by 8–9 days, whereas the grafts from the DENA-treated or tumor-bearing fish maintained their original sizes longer. Slight growth of some grafts taken from the fish treated with DENA for more than 4 weeks and from the tumor-bearing fish were apparent by 8–9 days.

Temperature Effects on Liver Tumorigenesis

Carcinogen Treatment at Different Temperatures

The combined results of 2 experiments on temperature effects on the incidence of liver tumors are shown in figure 2. When the fish were kept at a high temperature during and after the DENA treatment, tumor nodules were observed in all livers (HH-group). If the fish were transferred to a low temperature after treatment, small tumors that appeared during the treatment did not grow noticeably (HL-group). In the livers of these fish, mitotic figures were scarcely noted. On the other hand, liver tumors were rarely observed in the fish treated with the carcinogen at a low

TABLE 1.—*Behavior of liver grafts transplanted to anterior eye chamber*

Duration of the DENA treatment, wk	No. of donors	No. of recipient survivors at 13 days	Percent of grafts without remarkable degeneration at:[a]		
			4–5 days	8–9 days	12–13 days
0	3	11	27	0	0
1	4	15	27	13	7
4	5	21	48	10	5
6	3	15	47	33	7
4–9[b]	5	17	82	41	35

[a] The degeneration of the grafts was ascertained from observations under a dissection microscope.

[b] Donor fish were treated with 100 ppm DENA for 4 wk and then kept in normal tap water until 13 wk. All fish had tumor nodules.

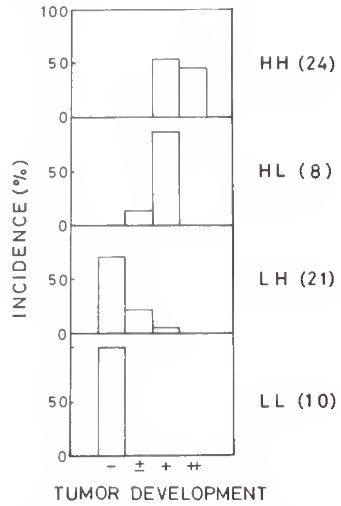

FIGURE 2.—Combined results of temperature effects on tumors induced by DENA. Degree of tumor development is ranked in the following 4 groups: ++ = fish with large tumor nodule; + = fish with small tumor nodule; ± = fish with the liver with many histological changes, such as large necroses and a proliferation of nonparenchymal cells but with no tumors; − = fish with the livers similar to those in controls. *Numbers in parentheses* indicate No. of fish observed histologically.

temperature. Only necroses and other histological changes were found when the fish were kept at a high temperature after the DENA treatment (LH-group). Livers of fish in the LL group also exhibited necroses. [A portion of our data has been reported (*6, 7*).]

Labeling and Mitotic Indexes of Liver Cells in Fish Held at High Temperature

The changes in LI and MI of DENA-treated liver cells are shown in figure 3. After 1 week of treatment, LI of parenchymal and nonparenchymal cells were at the control level, and no histological changes except for small necroses were observed. However, after 2 weeks LI increased rapidly and stayed at high levels during treatment. The labeled parenchymal cells could be classified into 2 groups: 1) normally stained cells and 2) basophilic cells. The latter type exceeded the former in number 3 weeks after treatment. Immediately after the DENA treatment was terminated, the LI decreased and returned to control levels by 13 weeks. Only in tumor nodules, however, were the LI maintained at high levels at 11–13 weeks. Moreover, MI increased with the increase of LI during the early period of treatment with the nitrosamine (fig. 3B), which indicates that the increase of LI was accompanied by the mitoses of the liver cells (*8, 9*).

Labeling Index of Liver Cells From Fish Held at Low Temperature

Changes in LI of hepatic parenchymal cells during and after the DENA treatment at 7±1° C are illustrated in figure 4. When the nitrosamine was administered at a low temperature, labeled cells were rarely observed and the index was zero. No histological changes appeared. When the fish were transferred to 25±2° C after treatment, the LI increased slightly. Most of the liver cells became eosinophilic, although small areas of the liver were basophilic at the higher temperature. At 13 weeks, 2 of 7 fish in the LH group had small tumor nodules in their livers. In another, necrosis and proliferation of the nonparenchymal cells were observed.

In the LL group, the LI remained at an extremely low level. However, necroses were observed in some fish after they were transferred to normal tap water, even if they were kept at 7±1° C. The cytoplasm of hepatic cells was slightly stained with eosin at 13 weeks, but the basophilic change was never seen.

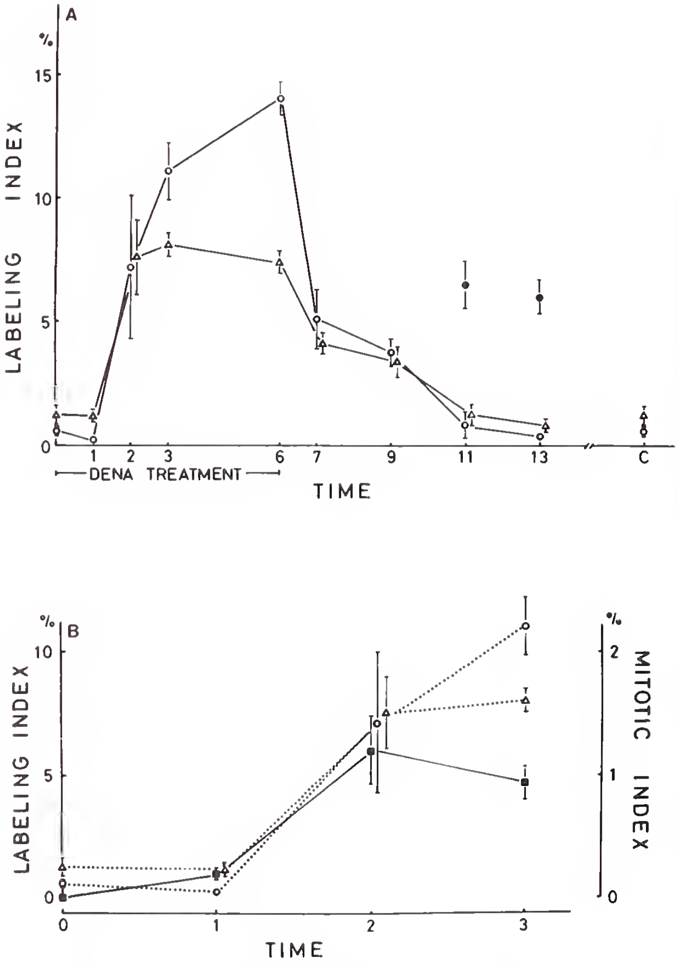

FIGURE 3.—Changes in LI and MI of liver cells during tumorigenesis. A) Changes in LI: *Each point* shows mean LI±SE from 5 to 7 fish. ——○—— = hepatic parenchymal cells; ——△—— = nonparenchymal cells; and ——●—— = cells in tumor nodules. B) Changes in MI and LI during the early period of DENA treatment. ——■—— = MI; ······○······ = LI of parenchymal cells; and ······△······ = LI of nonparenchymal cells. The LI is rewritten from the upper figure (*10*). Time is in weeks. Figure is reproduced with permission of the publisher (*10*).

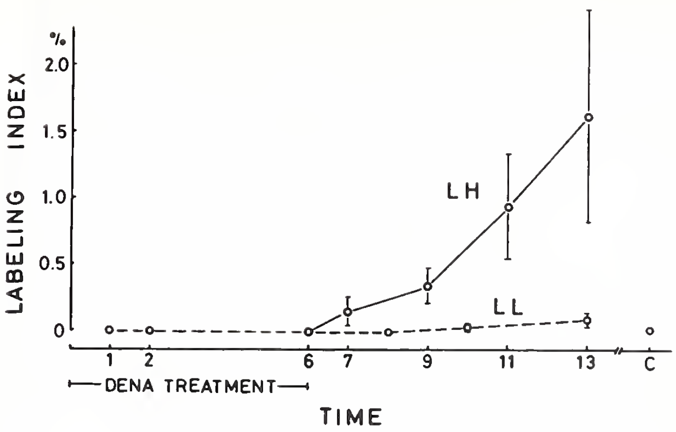

FIGURE 4.—Changes in LI of liver parenchymal cells during and after DENA treatment of 7± 1° C. Fish were transferred to normal tap water after treatment and kept at 25± 2° C (LH) or at 7± 1° C (LL). *Each point* shows the mean LI± SE from 3 to 5 fish. Time is in weeks.

Partial Hepatectomy in Medaka and Diethylnitrosamine Treatment

Changes in Mitotic Index of Liver Cells After Partial Hepatectomy

In this experiment, we dissected 30–70% of the liver tissue by weight of most fish. They died within a few days when the intestine herniated through the incision. Changes in MI in liver cells after the partial hepatectomies are shown in figure 5. One day after the operation, many erythrocytes were observed at the dissected portion. The hepatocytes adjacent to the wound were eosinophilic. Two days afterward, mitotic figures were observed around the dissected part and frequently throughout the liver by 3–4 days. A maximum MI of more than tenfold that of the control level was obtained at 4 days. Hepatocytes at the regenerating stage were basophilic without disruption of their normal arrangement. At 5 days, the dissected part of the liver was covered with connective tissue cells in some fish; the MI decreased gradually.

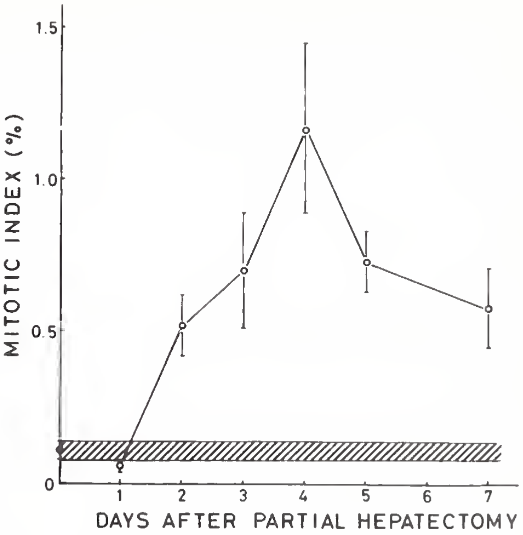

FIGURE 5.—Changes in MI after partial hepatectomy in medaka. Fish were treated with 2×10^{-4} *M* colchicine solution for 4 hr. *Each point* shows the mean MI± SE from 5 to 12 fish. More than 1,000 liver cells were observed/fish. *Hatched area*= mean MI± SE in sham-operated fish.

Nitrosamine Treatment After Partial Hepatectomy

The results of the DENA treatment after the partial hepatectomy are summarized in table 2. The fish which were moribund after the operation died within 3 days before treatment with DENA was started. The survivors were sensitive to sound, light, and any vibration, and had little appetite during the first 2–3 weeks.

About 40–70% of the liver (in weight) was removed by partial hepatectomy in this experiment. In groups C, D, E, and F, the ratios of liver weight to body weight in percent were: 2.8±0.2, 2.5±0.2, 4.0±0.3, and 3.8±0.3, respectively. Little difference was noted between the partially hepatectomized and the sham-operated groups. This fact suggested the complete recovery from the surgery. However, most of the fish treated with DENA after the partial hepatectomy had lumpy livers in contrast with the smooth

TABLE 2.—*Effect of partial hepatectomy on liver tumorigenesis before DENA treatment*

Group	Hepatectomy	DENA		Time of killing, wk	No. of fish	Stage of tumor development[a]			Percent of tumor-bearing fish
		Dose, ppm	Duration, days			1	2	3	
A	Partial	100	7[b]	11, 15[c]	12	9	2	1	75
B	Sham	100	7[b]	11, 15	14	8	3	3	58
C	Partial	100	4	11	7	3	1	3	43
D	Sham	100	4	11	9	2	0	7	22
E	Partial	200	4	6	12	5	4	3	42
F	Sham	200	4	6	8	4	4	0	50

[a] Values indicate No. of fish in the respective categories. 1= fish with tumor foci or nodules in their livers; 2= livers showed histologically abnormal features, increase of bile ducts, infiltration of nonparenchymal cells, etc.; 3=fish with livers similar to those of controls or with necroses.

[b] The DENA was added to physiological salt solution; fish in other groups were exposed to the carcinogen in tap water.

[c] No differences in tumor development were found between the fish killed at 11 wk and those at 15 wk; therefore, the combined data are given.

livers of sham-operated fish. The fish in groups A and B had grossly obvious tumors in their livers, whereas in the other groups, the tumors were detected only after microscopic observations.

Tumor incidence increased slightly in groups A and C compared with those in groups B and D. However, the results obtained in groups E and F were not in good agreement. The histological alterations observed in the fish ranked in (±) were more severe in groups A, C, and E than in groups B, D, and F.

DISCUSSION

When comparing the histological changes observed in the livers of DENA-treated medaka with the development of liver tumorigenesis in rats (*13-17*) and trout (*18*), we found the process of liver tumorigenesis in the medaka was similar to that in rats and trout. The result obtained by DNA cytofluorometry (*8*) shows that the hepatic cell fraction containing hyper 4C in DNA content was closely related to the existence of neoplastic cell subpopulations, which also agreed well with the results observed in rats given this carcinogen (*19*) and in human tumors (*20*). We believe that research on the medaka as an animal model will contribute to the investigations on chemical carcinogenesis.

Through the histological observations of DENA-induced liver tumors in the medaka, some have suggested that the precursor cells of the basophilic foci and the tumors would be found in diffusely spread basophilic areas. The transformed cells may appear between the stage when the basophilic areas develop and spread through the liver and the stage at which the basophilic cell foci appear. The results of the cytofluorometric measurements of DNA during the course of tumorigenesis (*8*) and of the liver transplants to eye chambers also suggest that the qualitative change of hepatic cells occurred at 3-6 weeks of the DENA treatment.

One of the advantages of using the medaka in tumor research is that we can examine the temperature effects on tumor induction and growth. The tumor incidence among fish held at a high or low temperature was remarkably different. These temperature effects should be considered from two standpoints. The first concerns the number of damaged sites caused by DENA in the liver cells. Initial damage may accumulate to some degree even at a low temperature because the histological changes, such as focal necrosis, were observed in the fish treated with DENA at a low temperature. The second is whether the DNA synthesis and cell proliferation occur during the course of tumorigenesis. The changes in LI and MI indicated the elevated hepatic cell proliferation during the DENA treatment at a high temperature. The early increase of LI after the application of a nitrosamine has also been observed before the appearance of tumors in rat liver and kidney (*21, 22*). Also from the results of histological observations and DNA cytofluorometry, the cell divisions in the early stages of carcinogen treatment were considered as compensatory cell proliferation (*8*). The damage caused by DENA might be amplified by this DNA synthesis and cell proliferation. Moreover, the newly apparent cells might also be more susceptible to DENA. Thus the transformed cells probably developed more frequently at a higher temperature, which corresponds to the higher incidence of hepatic tumors.

At the low temperature, cellular damage may have accumulated during the DENA treatment with only a little resulting in tumors after the fish were transferred to 25±2° C. The LI increased gradually, and histological changes were observed only after the transfer to a high temperature. Considering these results, we believe that DNA synthesis and cell proliferation may be necessary in the induction and growth of tumors.

We performed the partial hepatectomies before treatment with DENA to ascertain whether the liver tumors could be induced with a short-term treatment after induction of cell proliferation in the liver. The medaka did respond to the partial hepatectomy and the maximum MI was obtained 4 days after the operation. Cell kinetics following surgery were studied extensively especially in rats (*23-26*). The time at which the mitotic activity reaches its maximum rate varies among animal species; e.g., in rats, it is approximately 28 hours (*23*), mice, 3 days (*27*), dogs, 3-6 days (*28*), and more slowly in lower vertebrates (*29*).

The proliferating cells after partial hepatectomy are known to be more susceptible to carcinogens, and elevated tumor incidences were obtained when the nitroso compounds were given at the peak of DNA synthesis in rats after the surgery (*30-32*). In our study, treatment with DENA was also started 3 days after the actual or sham operations when the MI began to increase in regenerating livers. Tumor incidences were slightly higher in the partially hepatectomized medaka treated with 100 ppm DENA than in the sham-operated fish. Though the results obtained from the groups treated with 200 ppm DENA were not in good agreement, we can at least conclude that the regeneration of liver tissue in the medaka proceeds along a general pattern as seen in other species and that this surgical procedure can be used in fish for research on liver tumorigenesis.

A cell line from a DENA-induced liver tumor in the medaka has been established recently, and the results obtained from the analysis of chromosome number and histochemical assay of tyrosineaminotransferase strongly supported the premise that the cultured cells were derived from the transformed hepatocytes (*33*). Moreover, inbred strains of medaka have also been established (*34*). The use of these strains may minimize the varieties of individual responses to carcinogens and overcome the immunological problems in tumor transplantation. These inbred strains and the cultured cell line will advance the further analytical research required on liver tumorigenesis in fish.

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DISCUSSION[1]

J. Rice: Thank you very much, Dr. Kator, for the difficult task of presenting another person's paper.

M. Lipsky: In general, I think the work presented is good and novel. I do have one comment and one question. Was that dose of DENA after the partial hepatectomy a single dose, or was it the prolonged treatment?

K. Kator: The medakas had been treated with the carcinogen only for 4 or 7 days.

Lipsky: After the partial hepatectomy, too?

Kator: Yes. First they were partially hepatectomized, and after 3 days treatment with the chemicals was started.

Lipsky: I have one other comment on that. In our rodent models and work with the partial hepatectomy model followed by carcinogen for initiation, the dose is usually given much earlier, i.e., 24 or 36 hours, not when the mitotic index is elevated, but when the rate of DNA synthesis is elevated.

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[1] Dr. K. Kator responded for Dr. Kyono-Hamaguchi.

I think if you repeated the experiment and checked the incorporation of [^{3}H]dThd to see if, in fact, it is the same as in the mammal with DNA synthesis occurring earlier, you might increase your tumor initiation and promotion by giving the dose at an earlier point, when DNA synthesis is maximal, and not mitosis.

E. K. Weisburger: This enhancing effect of the partial hepatectomy is similar to that seen in rodents, and, in that respect, fish and the rodent react alike.

H. Kraybill: You mentioned here that you had DNA synthesis and cell division that occurred during the treatment. Are there other biochemical parameters that you would see, such as enhancement of enzyme activity?

Kator: No. She only measured DNA synthesis and the mitotic index.

J. Klaunig: Although they did not appear in the table, were any tumors produced by partial hepatectomy alone?

Kator: I think Dr. Kyono-Hamaguchi has not examined that.

K. L. Hoover: I wonder if you would get a clearer effect of the partial hepatectomy by studying the livers earlier than 11 and 15 weeks. It seemed to me that in your chart that was the time at which the fish were killed and, with partial hepatectomy, it can hasten the development of tumors. I wondered if you were to go back to 5 or 6 weeks, you might see additional effects.

Also, one other question as to technique. Do you have any idea how many survivors Dr. Kyono had from this partial hepatectomy procedure?

Kator: Yes, I have. These are difficult because often the whole intestine comes out, and so they die. She used physiological saline. She told me that at first she must keep them quiet. So, after the operation, the medakas had to be carried "very quietly." You may enjoy that but even so, we wrote on the front of our incubator, "Don't make any noise." That is true, at least, for 1 or 2 days. After that they become stable.

Rice: One question about the interesting difference seen in animals treated at low or high temperatures. One possible explanation of that might be that the metabolism of the carcinogen simply does not occur at the lower temperature, which, of course, would have profound implications for the use of fish as environmental monitoring agents. Do you believe that that is a reasonable explanation?

Kator: Yes, it is.

Factors Influencing Methylazoxymethanol Acetate Initiation of Liver Tumors in *Oryzias latipes:* Carcinogen Dosage and Time of Exposure[1, 2]

Kazuko Aoki *and* Hiromichi Matsudaira[3]

ABSTRACT—Hepatic tumors were produced in the medaka (*Oryzias latipes*) kept at 25° C in aquaria after exposure to methyl azoxymethanol (MAM) acetate. The tumors, including trabecular carcinomas and cholangiomas, were observable after 2–3 months. A similar incidence, i.e., nearly 100%, was found after a long-time exposure to a low level (0.1 ppm for 120 days) and after a short exposure to a high level (10 ppm for 1 hr) of MAM acetate. The relationship between the level of MAM acetate (0–2.0 ppm) and tumor incidence was studied after exposure for 1 day. The incidence increased as the level of drug increased, although an apparent threshold was seen at low levels. The incorporation of [^{3}H]thymidine into the liver greatly increased over the period of 6–20 days after treatment with 0.5 ppm for 3 days. However, only a slight increase in incorporation was found when fish were treated continuously with a low level (0.1 ppm) from days 30 through 90. No reduction of tumor incidence was found when the carcinogen was given in 2 divided doses separated by various intervals, compared with a single exposure. — Natl Cancer Inst Monogr 65: 345–351, 1984.

Hepatic tumors can be initiated by treatment with chemical carcinogens, such as DENA (*1, 2*) and MAM acetate (*3, 4*), in the small aquarium fish medaka, *Oryzias latipes.* The usefulness of this fish for chemical carcinogenesis studies was stressed by others as well (*1–4*). A high incidence of liver tumors including trabecular carcinomas and cholangiomas was observed within 2–3 months after short-term exposure to high levels (0.5–10 ppm) of MAM acetate, a stable form in aqueous solution of MAM, the active element in the naturally occurring glucoside carcinogen, cycasin. Also, the same tumors could be detected within 5 months after a long exposure to a low level (0.1 ppm) of the drug with similar incidence (*3*). The neoplastic changes in the liver after exposure of the fish to MAM acetate were similar to those after exposure to DENA. However, initiation of hepatic tumors required less exposure time with MAM acetate than with DENA (*4*).

The MAM acetate–medaka system is a convenient procedure with which one can analyze basic problems of chemical carcinogenesis. Here we report the results on the dosage and time of exposure, dose–response relationship, dose fractionation studies, and incorporation of [^{3}H]dThd into the liver at different times after treatment with MAM acetate.

MATERIALS AND METHODS

Adult male and female 1-year-old medakas (*O. latipes*) bred at this Institute were used. Groups of 10 fish each were kept at 25° ± 1° C under controlled light conditions (14 hr light and 10 hr darkness) in plastic tanks containing 2 liters of well water to which MAM acetate (Ash & Stevens, Inc., Detroit, Mich.) was added at levels ranging from 0.1 to 10 ppm depending on the experiments. The fish were fed Tetramin (Tetra Werke Co., Melle, Federal Republic of Germany). The water was changed every 3 days. After exposure to MAM acetate for the indicated periods, the fish were transferred to water not containing the drug and kept in it for various periods. To prevent the outbreak of infectious disease of fish, we added methylene blue to the water at levels of 0.5–0.75 ppm for 2 weeks after the treatment. Survival was recorded during 60 or 90 days. The fish were usually killed at 60 or 90 days, fixed in Bouin's solution, and their body and liver weights were measured. Serial sections (5 μm thick) were made from paraffin-embedded blocks and stained with hematoxylin and eosin for microscopic examination.

In the experiments of the incorporation of [^{3}H]dThd, the fish were given ip injections of 2 μCi (10 μl) of [methyl-^{3}H]dThd (sp act, 23 Ci/mmol; The Radiochemical Centre, Amersham, England), with a microsyringe (Hamilton Co., Reno, Nev.), during, or at different times after, exposure to MAM acetate.

Two hours after the injection, the fish were killed and fixed in Bouin's solution. After 24 hours, the livers were removed and transferred into 70% ethanol. Most liver samples were dissolved in 1 ml of Soluene 350 (Packard Instrument Co., Inc., Downers Grove, Ill.), and their radioactivity was measured with a liquid scintillation spectrometer. The results were expressed in disintegrations per minute per milligram fixed liver. Autoradiograms were prepared from some liver samples by the standard method with the use of an emulsion (Sakura NR-M2, Konishiroku Co., Tokyo).

For the split-dose experiments, the fish were exposed to the first dose, maintained in the drug-free water for indicated periods, and then exposed to the second dose. They were killed at 60 and 90 days after exposure to the

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ABBREVIATIONS: DENA = diethylnitrosamine; MAM = methylazoxymethanol; ppm = parts/million.

[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Supported in part by a grant-in-aid (No. 56010016) from the Ministry of Education, Science, and Culture of Japan.

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TABLE 1.—*MAM acetate-initiated hepatic tumor incidences in the medaka*

Level of exposure			Percent survival at day:		No. of fish with tumors/ No. of fish examined at day:		
ppm	Hr	Day	60	90	60	90	150
0.0			89	67	0/16	0/35	0/7
0.1		120		81		0/10	23/29
0.5		10	58		7/7	10/12	
2.0		1	60		4/6	9/12	
10.0	1		63		8/9	10/15	

first dose. The results were compared with those of single exposures at the same and twice the concentration or exposure time.

RESULTS

Table 1, which is a summary of the general results of the experiments [*see* (*4*) for details], includes levels and duration of exposure, survivals at 60 or 90 days, and the number of fish with tumors per number of fish examined microscopically.

The survival of the fish after the indicated treatments was slightly decreased compared with that of control fish. Liver neoplasms including trabecular carcinomas and cholangiomas were observed in fish exposed to MAM acetate at different levels and for different periods. No tumors were found in the controls. The neoplasms appeared earlier after exposure to higher drug levels than after exposure to the lowest level. Observation for 60 days seemed to be sufficient for us to measure the incidence of tumors for exposure to levels above 0.5 ppm and for treatment times usually longer than 1 day. For a given yield of the tumor, the product of levels in parts per million times duration of exposure in days became smaller for higher levels of the drug.

Figure 1 shows the changes in the survival and in the incidence of tumors at 60 days (A) and 90 days (B) as a function of the level of MAM acetate; the exposure time was 24 hours. No decrease was observed in the survival for exposures below 0.5 ppm, whereas a substantial decrease was found at higher levels. The surviving fraction at 90 days was lower than that at 60 days.

No tumors were found for exposures at 0.25 ppm at 60 days. At 90 days, however, 2 of 41 fish examined had tumors. Among the groups receiving different doses, the fraction of tumor-bearing fish of those surviving at 60 days (Y) could be fitted either to a relationship of $Y=1.18(X-0.29)$ or $Y=1.44(X-0.19)^2$, with X being the level of the drug in parts per million. When a similar calculation was made with the results at 90 days, however, only a linear relationship, $Y=1.21(X-0.19)$, was obtained between the incidence of the tumor and the level of the drug. These results indicate that, in this particular fish, the incidence of liver tumors is increasing linearly with dose, rather than with a dose-squared relationship, after a certain level of threshold and within the levels of MAM acetate examined.

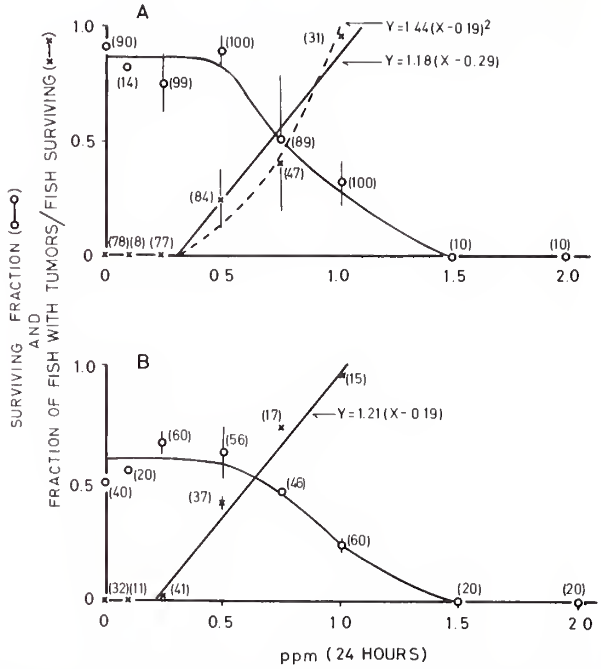


FIGURE 1.—Changes in the surviving fraction and in the incidence of tumors expressed in fraction of fish with tumors of those surviving at 60 days (A) and 90 days (B) after exposure to MAM acetate for 24 hr at indicated levels. *Numbers in parentheses* are the No. of fish tested (survival) and of fish examined (tumor incidence). *Vertical bar*=SE.

The relationship between length of exposure and tumor incidence is illustrated in figure 2. In this experiment, the fish were treated with 0.5 ppm of MAM acetate for 1, 2, 2.5, and 3 days and then kept for 60 days in well water at 25° C. When the incidence was plotted as a function of level in parts per million times duration of exposure in days, a relationship of $Y=0.68(X-0.045)$ was obtained.

If you compare the results shown in figure 1A with those in figure 2, you will note that a substantial decrease in the slope of the dose-effect relationship may take place after an exposure to a lower level but for a longer period compared with a short exposure at a high level for a given total dose.

In a typical experiment in dose-fractionation studies, no decrease in the incidence of hepatic tumors was found when

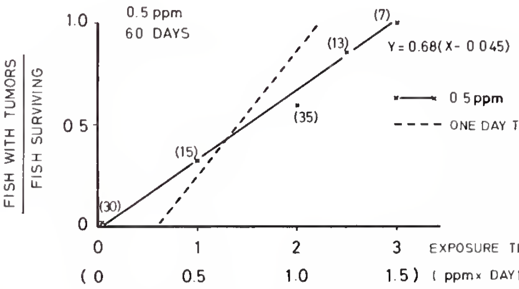


FIGURE 2.—Relationship between time of exposure in day (at 0.5 ppm) and tumor incidence. Fish were examined at 60 days. *Dashed line* is from figure 1A.

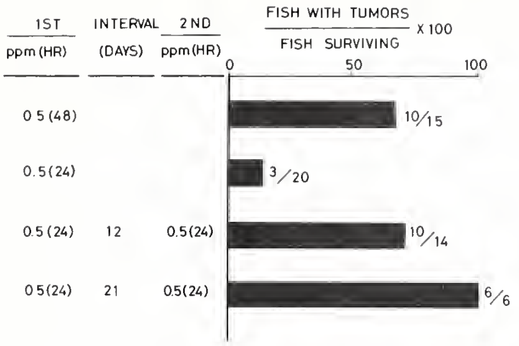


FIGURE 3.—Split-dose effect of MAM acetate on tumor incidence at 60 days.

the treatment was done as 2 equal exposures for 1 day with the indicated drug-free intervals, compared with a single exposure for 2 days (fig. 3). Similar results were obtained when the effects of exposure to 8 ppm for 1 hour were compared with those of 2 consecutive exposures at 4 ppm for 1 hour with intervals of 1 to 10 days.

To determine the relationship between the early responses to MAM acetate and carcinogenic developments, we examined the changes in the incorporation of $[^3H]$dThd into the liver of the fish.

Typical changes in the incorporation during the indicated periods after exposure to MAM acetate are illustrated in figure 4A–C. After exposure to 0.5 ppm for 3 days, a marked increase in the incorporation took place from about 1 week and continued until about 1 month; thereafter, a small increase in the incorporation was also seen. After this exposure, the tumor incidence at 60 days was nearly 100%. A heavy labeling confined to the nucleus of many liver cells was observed in autoradiograms of liver samples from fish 6 to 30 days after exposure to 0.5 ppm MAM acetate. A slight increase in $[^3H]$dThd incorporation was also observed by spectrometric analysis during a continuous exposure at 0.1 ppm MAM acetate.

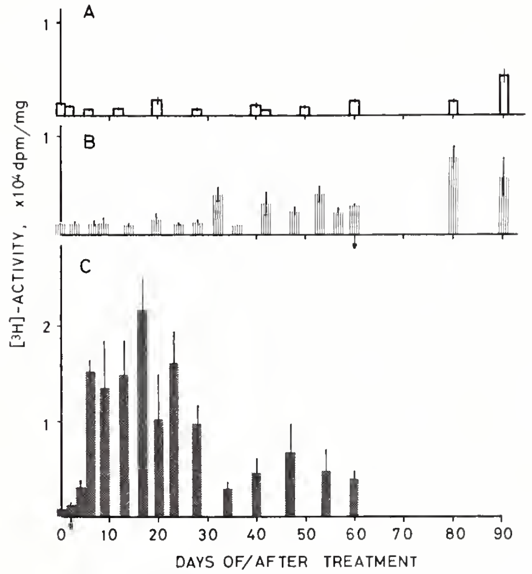


FIGURE 4.—Changes in the incorporation of $[^3H]$dThd into the liver during or after exposure to MAM acetate. A) controls; B) continuous treatment at 0.1 ppm for 60 days; C) 0.5 ppm for 3 days. *Vertical bar*= SE, *arrow*= the end of exposure.

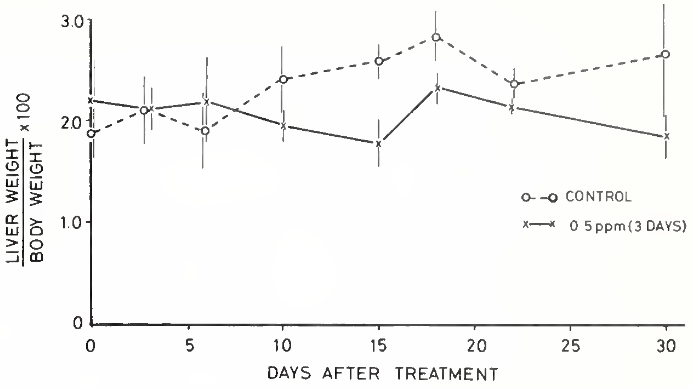


FIGURE 5.—Changes of the liver weight after exposure to MAM acetate at 0.5 ppm for 3 days (X———X); O- - - - -O = control; *vertical bar*= SE.

Inasmuch as the increase in the incorporation of $[^3H]$dThd into the liver reflects cell proliferation which may follow cell degeneration, the changes in the liver weight were examined after MAM acetate exposure. The results shown in figure 5 indicate a slight but significant decrease ($P<0.01$) in the liver weight during the period when a marked increase in the incorporation of $[^3H]$dThd was seen. This decrease was more marked after exposure to higher levels of the drug.

The early changes after MAM treatment were examined histopathologically as well. Figure 6 (A–E) shows the histology of liver samples from control fish and fish at 6 and 30 days after exposure to MAM acetate at 0.5 ppm for 3 days.

The two-layer arrangement of hepatic cells in muralia was not readily apparent by 6 days. The cell nucleus was enlarged, and the nucleolus became prominent (fig. 6B). Thirty days after exposure, the ordered cellular arrangement was almost totally lost (fig. 6C), and numerous minute nodules comprised of large cells were seen (fig. 6D). A small nodule consisting of cells with increased basophilic staining of the cytoplasm was also noted (fig. 6E).

DISCUSSION

The results presented in table 1 show that the medaka is a convenient animal model for testing the carcinogenic effects of water-soluble chemicals such as MAM acetate. Tumors can be produced in high yield within short intervals after various regimens of exposure, including short (1-hr) exposure to high levels (8–10 ppm) and long exposure (4 mo) to a low level (0.1 ppm). The type and histology of tumors produced did not vary greatly from those produced by DENA in the same species (*1, 2*). Therefore, this system may provide a means by which one can test and analyze the carcinogenic effects of chemicals.

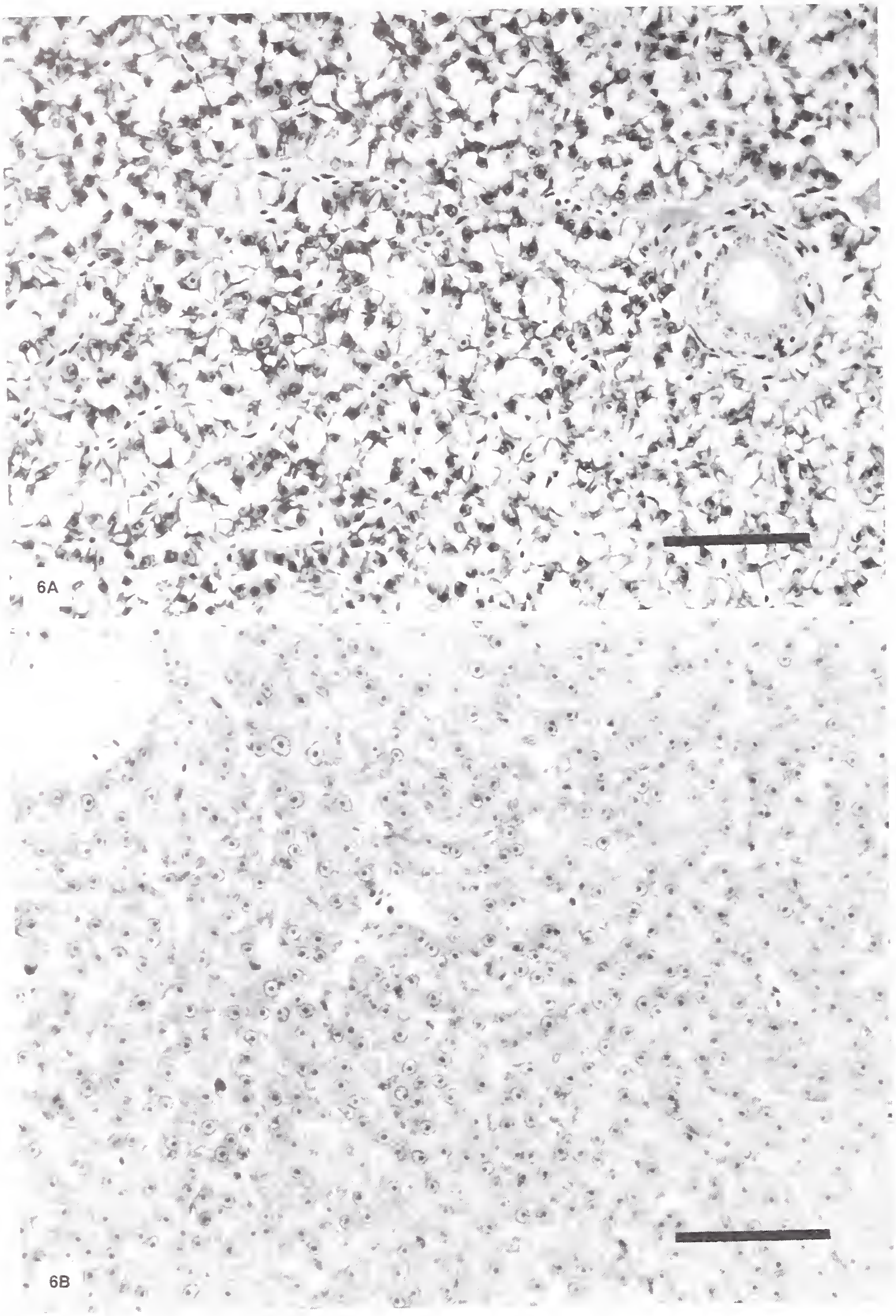
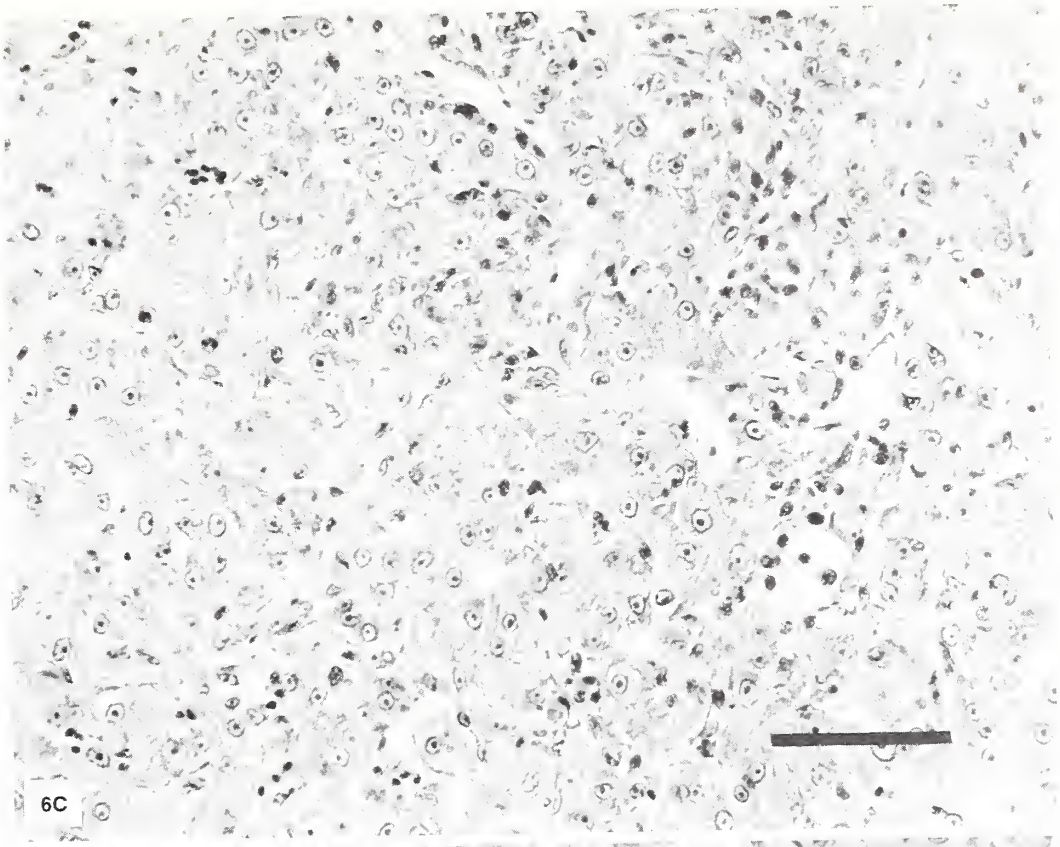


FIGURE 6A–E.—Histology of liver samples from controls (A) and fish at 6 (B) and 30 (C–E) days after exposure to MAM acetate at 0.5 ppm for 3 days. *Arrows* (E) show a small nodule. Hematoxylin and eosin. *Bar* = 50 μm.



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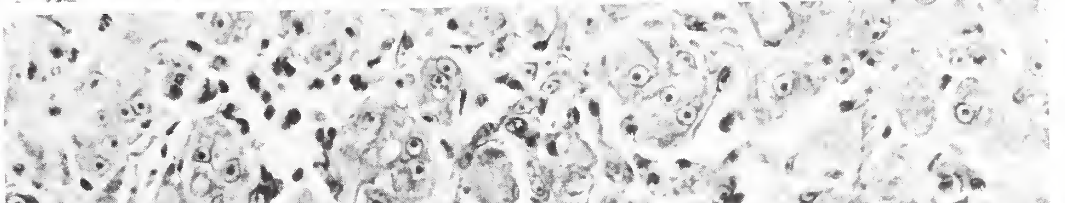

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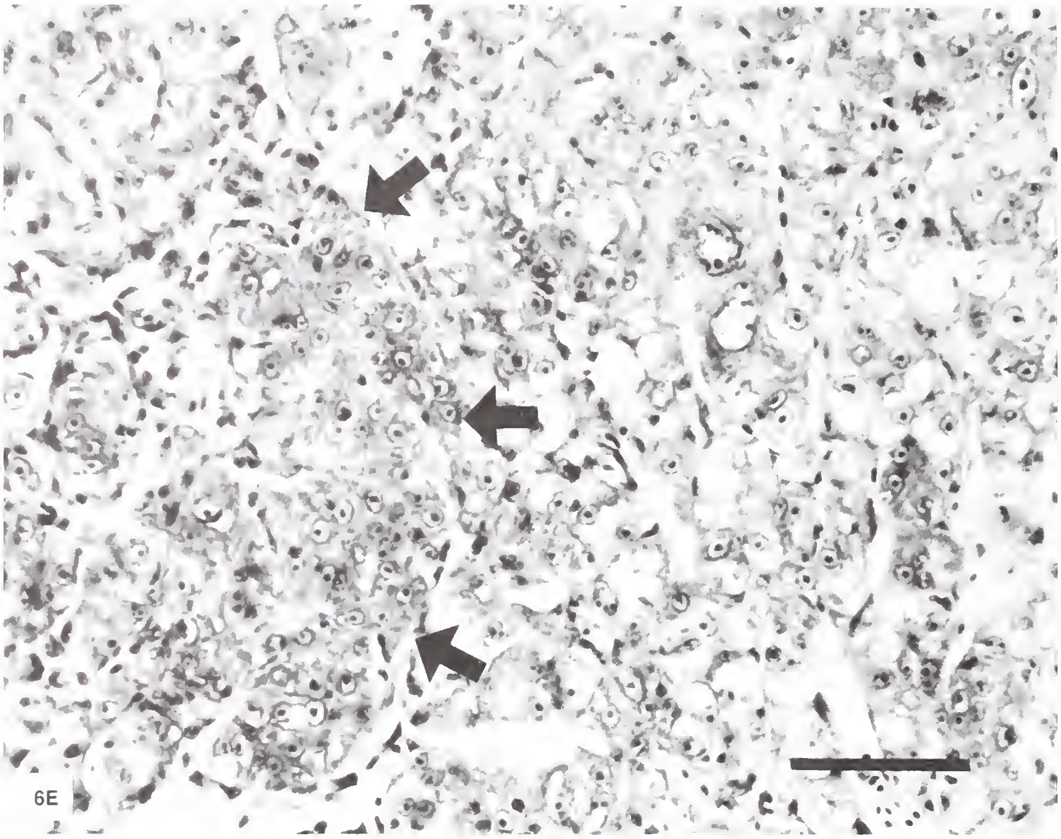

The dose–response relationship of chemical carcinogens was studied also in aquarium fish. Grieco et al. (*5*) obtained a linear and probably a no-threshold relationship in rainbow trout fed dimethylnitrosamine. The results reported by Hendricks (*6*) show a probable supralinear (upward convex) relationship with no threshold in aflatoxin B_1-treated 21-day-old rainbow trout embryos. The findings of Ishikawa and Takayama (*7*) on medaka treated with DENA could be interpreted as showing an apparent threshold. The response noted in our experiments could be fitted best to a linear relationship. The presence of a threshold was interpreted as apparent because exposure to a lower level but for longer periods could produce tumors.

The results shown in figure 2 and summarized in table 1 may indicate a possible decrease in the tumor incidence for a given total dose when the exposure was protracted. They suggest the possible presence of a dose-rate effect, which has been documented in radiation carcinogenesis (*8*), in chemical carcinogenesis as well. However, this point needs more careful study.

We are not aware of other dose-fractionation studies on aquarium fish. Swann et al. (*9*) observed a considerably decreased incidence in dimethylnitrosamine-initiated kidney tumors in the rat when the drug-free time of 8–32 days was allowed between the first and second doses, whereas this effect was not seen at shorter intervals. Yuhas (*10*) observed a recovery from urethan-initiated carcinogenic injury to the mouse lung at a high dose. When the dose was low, however, recovery was not seen. As the results in figure 3 indicate, no recovery of the carcinogenic effects of MAM acetate occurred.

In her studies of the changes in the labeling indexes throughout liver carcinogenesis by DENA in the medaka, Kyono et al. (*11*) detected a marked increase in [^{3}H]dThd labeling in parenchymal and nonparenchymal liver cells 2 weeks after DENA administration, long before the appearance of tumors. This increase was reported to be temporary because it disappeared rapidly (within weeks) when the drug treatment was discontinued and the fish were returned to well water. These findings are consistent with ours for MAM acetate. That proliferation is necessary both for fixation and expression of malignant transformation of cells is well-known (*12*). One can assume that the changes in cell proliferation initiated by the first dose of MAM acetate may alter the cellular response to the second dose and be partly responsible for the lack of recovery in split-dose experiments, although other explanations are also possible.

The increase of [^{3}H]dThd uptake in the liver may provide a simple and rapid means for testing chemical carcinogens. Investigators' attempts to detect unscheduled DNA synthesis have not been successful. The early histological changes as shown in figure 6 may also be useful in preliminary screening of chemical carcinogens, although the development of more refined methods, such as the detection of enzyme-altered foci, would have greater diagnostic value.

Recently, inbred strains of medaka have been established by Hyodo-Taguchi (*13*) in our laboratory. She examined the difference in the sensitivity to MAM acetate between these strains. They could be divided into 3 groups: sensitive, intermediate, and resistant. Embryos of these strains differed also in the sensitivity to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and UV (*14*). Detailed characterization of these strains in relation to chemical carcinogenesis is now in progress.

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DISCUSSION

G. Bailey: This is just a comment on the possibility of using [^{3}H]dThd incorporation as a measure of precarcinogenesis. Of course, any compound that causes simple mitotic stimulus would be picked up by that kind of test.

G. Streisinger: I think that Dr. Aoki's work illustrates an extremely important and unique use to which fish can be put that I am really glad was brought out at this meeting because I would like to emphasize it again to the consensus committee and to the organizers of this meeting. The nature of the dose response, I think, is crucially important for theoretical and regulatory purposes. The kind of experiment described by Dr. Aoki can be done uniquely well with medaka and with a number of other strains of fish because they have low background levels and because experiments are possible with large numbers. For these experiments, I believe it is most important for us to realize that one does need inbred strains, uniform strains, such as medaka, zebra fish, or others, because otherwise genetic variability in the strain would obscure the nature of the dose response. Here is a point which I hope the consensus committee will make. This is a research area for which fish are uniquely useful.

Now, one other minor comment to Dr. Aoki. I think it would be especially important to measure dose response in a slightly lower range and then to analyze it by looking at a plot of logarithm of effect versus logarithm of dose, and, for the range you have studied, it might be also interesting to plot the logarithm of the fraction that does not have tumors against dose. That makes analysis easier because at 100% you cannot do more, and it is hard to see that. Thank you very much for these excellent results.

K. Hoover: I would like to second what Dr. Streisinger has just said. This is a highly sophisticated set of experiments which you have done, and their need is great in this field.

I would like to note that your choice of medakas that are 1-year-old interests me. Most people go to the few-week-old or few-month-old animals to begin the testing, but you have chosen the year-old. I wondered what your reason was for doing that. It appears that they are just as sensitive when they are halfway through their life-span.

K. Aoki: I am now trying the use of younger fish.

SESSION V

Metabolic Handling of Xenobiotics by Fish

Chairman: Lionel Poirier, Ph.D.

Biotransformation of Chemicals by Fish: An Overview [1, 2]

John J. Lech *and* Mary Jo Vodicnik[3]

ABSTRACT—Evidence accumulated in recent years indicates that many species of fish possess most of the hepatic drug-metabolizing enzyme activities that have been demonstrated to exist in higher vertebrates. Although much of the early work with fish involved in vitro studies, it is now apparent that fish are capable of many phase I and II biotransformation reactions in vivo as well, but the rate and pathways for a given compound may vary widely among species of fish. In several instances, it has been demonstrated that the rate of biotransformation of certain compounds is appreciable. Biotransformation may be important in protecting fish from the toxic effects of certain chemicals by converting them to less biologically active chemicals. On the other hand, evidence is mounting that certain species of fish can react in such a way in vivo so as to produce active carcinogens from chemicals which are not themselves carcinogenic. — Natl Cancer Inst Monogr 65: 355–358, 1984.

Although the biotransformation of xenobiotic chemicals has been intensively studied in mammalian species for many years, comparable investigations in aquatic species lagged behind mammalian research, and it is only in the last 10 years or so that we have begun to appreciate the importance of biotransformation among fish and other aquatic species. In vitro studies in which fish tissue fractions and model substrates were used demonstrated phase I and phase II biotransformation reactions, although the absolute level of enzyme activity varied considerably among species (*1–6*). These studies indicated that fish have the capacity to biotransform a variety of substrates, but, generally, the rates observed were lower than those in many mammalian species. Monooxygenase activity associated with isolated hepatic microsomes of several species of fish has been demonstrated to be sensitive to inhibition by carbon monoxide and responsive to mammalian monooxygenase modulators such as α-naphthoflavone and metyrapone (*7–9*). A reasonable amount of evidence exists to indicate that phase I reactions demonstrated in fish in vitro are catalyzed by the microsomal cytochrome(s) P-450 system, which has been extensively investigated in mammals (*7, 10, 11*). Although the purification and characterization of cytochrome(s) P-450 from fish tissues have not approached the sophistication observed with comparable mammalian systems, it would appear that the substrate specificities are similar to those seen in mammalian systems (*12*). That both benzphetamine-*N*-demethylase and ethylmorphine-*N*-demethylase activities have been demonstrated in hepatic microsomes of fish suggests that a system comparable to the cytochrome(s) P-450 system of mammals exists, but evidence indicates that this system in fish appears to be refractive to induction by drugs and chemicals which produce phenobarbital-type induction in mammals (*12, 13*). On the other hand, monooxygenase activity toward ethoxyresorufin (de-ethylation), a highly specific substrate for the polycyclic aromatic hydrocarbon-inducible form(s) of cytochrome P-450, designated cytochrome P_1-450, has been shown to be present in hepatic microsomes from several species of fish (*12*). This latter form of P-450 is inducible by 3-methylcholanthrene and other inducers of this class, including polycyclic aromatic hydrocarbons and several co-planar polychlorinated biphenyl congeners (*12, 14, 15*). Phase II reactions have been demonstrated in vitro with tissue preparations from various fish species; it is clear that glucuronyl transferase, sulfotransferase, glutathione-*S*-transferase, and epoxide hydrolase activities are, at least qualitatively, similar to those found in mammals (*8*). Although investigations in vitro have been extremely useful in characterizing the enzymes involved in the biotransformation of chemicals in fish, one can readily realize that an area of equal or greater importance is concerned with the functional significance of these biotransformation reactions in vivo. It is important to note that almost every biotransformation reaction (phase I and phase II) that has been described in vitro in fish has occurred in vivo during exposure of live fish to various chemicals and pesticides, and metabolites arising from biotransformation reactions have been identified in fish tissues including liver, bile, and urine (*16, 17*). The information presented in table 1 illustrates a partial listing of biotransformation reactions which have been demonstrated in various fish species in vivo with specific xenobiotic chemicals.

From a functional point of view, biotransformation reactions can significantly influence the biological properties of xenobiotic chemicals, depending on the qualitative nature of the reaction and the rate at which it occurs in vivo (*16*). Table 2 illustrates several important toxicokinetic parameters which are influenced by biotransformation reactions. Whereas these important areas have not

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[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Supported in part by Public Health Service grant 01080 from the National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, North Carolina, and by grant 01985 from the NIEHS Aquatic Biomedical Research Center Program.

[3] Department of Pharmacology and Toxicology and the NIEHS Freshwater Biomedical Research Center, The Medical College of Wisconsin, Milwaukee, Wisconsin 53226.

TABLE 1.—*Biotransformation reactions demonstrated in vivo by several fish species*

Biotransformation reaction			
Phase I	Phase II	Species	Chemical
O-Dealkylation		Fathead minnow	*p*-Nitrophenylethers
		Rainbow trout	Pentachloroanisole Fenithrothion
N-Dealkylation		Carp	Dinitramine
Oxidation		Mudsucker, sculpin	Naphthalene, benzo[*a*]pyrene
		Coho salmon	Naphthalene
		Rainbow trout	Methylnaphthalene
		Carp	Rotenone
		Mosquito fish	Aldrin, dieldrin
Hydrolysis		Catfish, bluegill	2,4-Dichlorophenoxyacetic acid esters
		Rainbow trout	Diethylhexylphthalate
		Pinfish	Malathion
		Mosquito fish	Parathion
	Acetylation	Dogfish shark	Ethyl-*m*-aminobenzoate
		Rainbow trout	Ethyl-*m*-aminobenzoate
	Glutathione conjugation	Carp	Molinate
	Taurine "	Flounder	2,4-Dichlorophenoxyacetic acid
	Sulfate "	Goldfish	Pentachlorophenol
	Glucuronide "	Rainbow trout	Pentachlorophenol
		Rainbow trout	3-Trifluoromethyl-4-nitrophenol
		Goldfish	Pentachlorophenol
	Glycine "	Flounder	Aminobenzoic acid

TABLE 2.—*Consequences of biotransformation of chemicals*

Modulation of blood and tissue concentrations
Control of bioaccumulation and persistence (half-life)
Conversion of a toxic compound to a less toxic one
Conversion of a nontoxic compound to a toxic one

been extensively studied in fish, we (*16*) have evidence that the biotransformation of certain chemicals in fish may be critical to several, and possibly all, of these parameters. The intimate details of the biotransformation of most carcinogens in fish remain to be elucidated, but apparently the reactions leading to carcinogenic metabolites of benzo[*a*]pyrene, aflatoxin B_1, and dimethylnitrosamine occur in fish in vivo based on metabolism studies and demonstration of their carcinogenicity (*18-20*).

Central to the topic of the comparative aspects of chemical carcinogenesis are the qualitative and quantitative aspects of the biotransformation and toxicokinetics of a given chemical carcinogen in various species of fish when compared with similar parameters in mammalian models. Whereas these factors which affect the concentration of an active carcinogen at the target site are important in influencing the response of a tissue to a carcinogen, the presence or absence of a target organ among species and its inherent sensitivity are equally important, especially if the data are to be used for extrapolation purposes.

An understanding of carcinogen biotransformation is pertinent to the overall goal of this Symposium, inasmuch as the rate and pathway of biotransformation of a carcinogen in a given species and target organ sensitivity are important determinants of the dose-response relationship of the biological end point, which is tumor formation. Clearly, the current literature reveals that biotransformation reactions play a major role in the overall behavior of certain chemicals in fish and that the manipulation of the rates of these reactions by inducers or inhibitors of biotransformation can significantly affect important toxicokinetic parameters, such as steady-state tissue levels, persistence, and metabolite patterns.

Although the scientific state of the field has moved rapidly in the past 5 years, we know less about the toxicokinetic behavior of carcinogens in fish than we do about other chemicals. Probably the best studied carcinogen in regard to biotransformation is benzo[*a*]pyrene, but carcinogenesis studies involving this chemical in fish are sparse. Conversely, aflatoxin B_1 has been extensively investigated with respect to tumor formation in fish (*21*), but intimate details of its biotransformation, toxicokinetics, and their relationship to the high sensitivity of trout to this hepatic carcinogen await further investigations. If small fish species are to be used in a bioassay program for carcinogens, then one must cope with the problem of extrapolation to man from a species which may be more distant from man than currently utilized rodent models. Figure 1 summarizes some of the factors that bear on the sensitivity among species to carcinogens. Important considerations are 1) the toxicokinetic and metabolic bases for the dose-response relationship, i.e., the delivery system; 2) organs which mammals and fish have in common and those which do not exist in common, e.g., gills, swim bladder, lung; and 3) the inherent sensitivity of common organs or cells to an active carcinogen, based on rates of DNA repair and response to promoters.

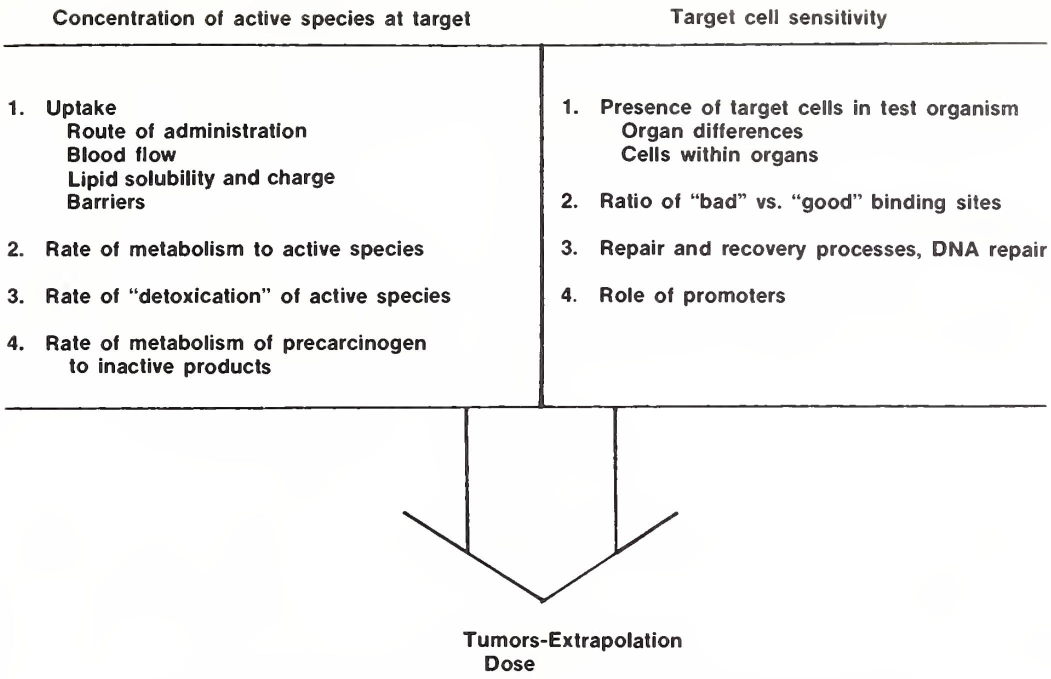
Concentration of active species at target
Target cell sensitivity
1. Uptake
Route of administration
Blood flow
Lipid solubility and charge
Barriers
2. Rate of metabolism to active species
3. Rate of "detoxication" of active species
4. Rate of metabolism of precarcinogen to inactive products
1. Presence of target cells in test organism
Organ differences
Cells within organs
2. Ratio of "bad" vs. "good" binding sites
3. Repair and recovery processes, DNA repair
4. Role of promoters
Tumors-Extrapolation
Dose

FIGURE 1.—Summary of factors pertinent to species sensitivity to carcinogens.

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DISCUSSION

L. Poirier: Has metabolic activation, when fish microsomes are used, been linked to mutagenesis in tests similar to the Ames?

J. J. Lech: Yes, I am quite sure it has. We have never done it, but I have seen people take S-9 from control animals or animals pretreated with Aroclor 1254 and show that they can activate carcinogens in the Ames test. I think 4 or 5 investigators have shown that now.

Poirier: Do you know if the activity is comparable to that of mammalian cells?

Lech: I really cannot answer that. We have not done that comparison, but in some cases it may be higher and in others lower, depending on the compound. I have not done the Ames test. If you look at the whole animal, biliary excretion of metabolites in the rainbow trout exposed to, e.g., benzo[*a*]pyrene or 2-methylnaphthalene, you find milligram quantities of metabolites produced by the animal. Therefore, I think that the rate of biotransformation in fish is generally not extremely slow; I think it depends on the compound and species.

K. L. Hoover: How would you deal with the whole problem of dosage in fish? That is, when you put a compound into the water, you really cannot be certain how much is getting into the fish. Is injection the only acceptable way?

Lech: Well, this is a two-faceted problem. I do not have any problem in exposing a fish or an egg to a compound because we measure the amount that enters the organs. You worry about dose because you have to measure whether it gets in or not. If you remember, Leif Marking was talking about the pH effect, and Dr. Rice mentioned checking the amount of aflatoxin that gets into the fish egg before you call it a negative response. The problem I have is determining how you extrapolate dosage to a risk assessment, i.e., exposure to man? I do not have a problem with fish responding to chemicals through biotransformation, the end point being a tumor. What I have a problem with is how to take that experimental situation in which you are creating dosages and extrapolate that to other species. How do you compare exposure of a fish egg with exposure of man to a carcinogen in his diet?

What I am saying is that you could always control for the fact that if you put a compound in with the fish egg and get a negative response, you can measure whether the compound got into the embryo or not. If it gets in, and it does not produce a tumor, that is fine. Target organ concentrations versus route administration are critical. Again, how do you extrapolate?

Hoover: You always encounter this problem with the rodent system, in that researchers are feeding or administerint high levels of the test compounds. It goes back to the same question. How can you extrapolate to man the conditions of a model system when you are overwhelming the system.

Lech: I think that your question is more a philosophical question than a scientific one. Investigators look into carcinogenesis in fish for several reasons. In studies on 1) basic mechanisms, extrapolation to other species is *relatively* unimportant; 2) protection of aquatic resources, extrapolation to mammalian species is *not* important; and 3) risk assessment for human cancer, the use of fish as tools presents many of the same problems in extrapolation to man as does the use of other experimental animals. Do we use the most sensitive species and err on the safe side or demand firm data in man?

Hoover: Yes, it also depends on exactly what it is that you are trying to determine when you are using the fish. I mean that the fish can be used as a first-tier screening test rather than a hard-and-fast extrapolation.

Lech: I agree with that; that is my business. I would not fight against that, but I am just pointing out for the purposes of this Conference that we have a long way to go before we see the total picture.

J. R. Bend: Dr. Lech, I agree with much of what you are saying, but in trying to get some extrapolatable value, if you will, I think we can think of effective dose. Perhaps one effective dose to consider might be the specific amount of alkylated DNA. It is thought to be the product of a reaction between the ultimate carcinogen and a specific base. This might have the advantage of being usable in fish, no matter whether it is an embryo exposure, a water exposure, or a mammalian system.

Lech: I totally agree because you can take that point a bit further. People have wanted to look at the levels of X, Y, and Z that are formed metabolically in, e.g., liver tissue, and say that this benzpyrene 4,5 oxide, or whatever compound you wish, is a toxic species. I think that is not the correct approach to take. I believe that you should do what you have just said. First measure the actual amount of DNA damage and then relate that to any given exposure mode. That would be an end point taken back from the tumor.

Heterogeneity of Hepatic Aryl Hydrocarbon Hydroxylase Activity in Feral Winter Flounder: Relevance to Carcinogenicity Testing[1, 2]

John R. Bend,[3, 4] Gary L. Foureman,[3, 4] Zvi Ben-Zvi,[3, 5] *and* Phillip W. Albro[6, 7]

ABSTRACT—Hepatic microsomes from winter flounder (*Pseudopleuronectes americanus*), treated with polycyclic aromatic hydrocarbon (PAH) inducers, had elevated activities of benzo[*a*]pyrene hydroxylase (AHH) and 7-ethoxyresorufin deethylase (7-ERD). When electrophoresed, they showed a novel or enriched polypeptide species with a monomeric molecular weight of approximately 57,000. These results are consistent with inductive responses already well-characterized in several mammalian and fish species. However, when we studied the urinary clearance of 4-chlorobiphenyl in untreated flounder, wide variations (up to twentyfold) among fish were noted. Subsequent in vitro analysis of AHH and 7-ERD activities in liver demonstrated wide variations in these monooxygenase activities in flounder caught near Mount Desert Island, Maine. In some instances, AHH activities in these feral flounder were as high as those in PAH-induced fish. Based on the response of AHH activity to 7,8-benzoflavone (ANF) added in vitro, flounder could be divided into 2 groups; one had high hepatic AHH activity which was inhibited by ANF, the other had low AHH activity which was enhanced by ANF. Examination of a large number of winter flounder (>400 total) over 4 experimental seasons demonstrated this variability of hepatic AHH activity to be a recurrent characteristic of the flounder population in waters around Mount Desert Island. The hepatic AHH activities did not correlate well with any physical parameter of the fish (e.g., liver, gonad or body weight, length, or sex) or the cytochrome P-450 content of the hepatic microsomes. Our attempt to evaluate the AHH activity (high vs. low) of individual fish in vivo by urinary clearance of antipyrine was unsuccessful, due to the excretion of large amounts of unchanged antipyrine through the gills. Similar studies were performed with another marine teleost, *Fundulus heteroclitus.* Of the approximately 200 *Fundulus* examined, almost all had AHH activity inhibited by ANF; in some experiments, hepatic 7-ERD activities were further increased after treatment with the potent PAH-type inducer 3,4,5,3′,4′,5′-hexachlorobiphenyl but only about twofold. Collectively, these data are consistent with PAH-type induction of the hepatic cytochrome P-450-dependent monooxygenase system in some feral marine teleosts (in winter flounder and *Fundulus*) from Maine by environmental contaminants or food constituents. It is not known whether these chemicals are of natural or anthropogenic origin. The variation in the response appears to be related to individual exposure level (dose) or sensitivity, or both. In any event, the possibility of heterogeneity of hepatic monooxygenase activities in teleost species should be considered in development of experimental models for carcinogenesis testing in fish. — Natl Cancer Inst Monogr 65: 359–370, 1984.

Accurate assessment of the toxicological properties (including carcinogenicity) of industrial chemicals and their by-products and of environmental pollutants in a cost-effective manner is a major problem of our high technology society. The number of compounds involved increases every year. Consequently, researchers are trying to develop more economical or more sophisticated, or both, in vivo and in vitro tests for determining chemical toxicity. They are also interested in developing sensitive biological end points that can be used as sentinel systems for the presence of toxic chemicals in the environment. Obviously, in this regard, the etiology of the biological response must be known in detail for the monitoring to be effective. Fish have been suggested both as in vivo systems for testing for carcinogenesis and as sentinel species for the detection of certain chemical classes of environmental pollutants; both suggestions are practical for certain reasons.

It is now widely recognized that many chemicals which show mutagenic or carcinogenic activity in in vivo or in vitro test systems must first be metabolically activated to the ultimate carcinogen or mutagen, i.e., the metabolite (or metabolites) that react with critical macromolecules and initiate the process eventually leading to carcinogenesis or

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ABBREVIATIONS: PAH = polycyclic aromatic hydrocarbon(s); AHH = aryl hydrocarbon hydroxylase; 7-ERD = 7-ethoxyresorufin deethylase; HCB = 3,4,5,3′,4′,5′-hexachlorobiphenyl; 4-CB = [^{14}C]4-chlorobiphenyl; HEPES = 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; FU = fluorescence unit(s); ANF = 7,8-benzoflavone; BND = benzphetamine demethylase; SDS–PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BNF = 5,6-benzoflavone; DBA = 1,2,3,4-dibenzanthracene; HPLC = high-performance liquid chromatography.

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mutagenesis. Thus any discussion of the use of fish species for carcinogenesis testing or as environmental monitors must include their ability to metabolize chemicals. Both marine and freshwater fish contain virtually all the enzymatic pathways present in mammals for xenobiotic biotransformation [*see* (*1-3*)]. The cytochrome P-450-dependent monooxygenase system, essential for the oxidative metabolism and activation of many procarcinogens, is present in the liver of every fish that has been studied adequately. This monooxygenase activity is increased with certain substrates, such as benzo[*a*]pyrene, by the administration of PAH-type compounds like 3-methylcholanthrene (*4, 5*), although the hepatic system of fish apparently does not respond to phenobarbital-type inducing agents (*6*). As in mammals, the large increase in hepatic microsomal AHH activity of fish after treatment with PAH-type compounds is related to the presence of one or more novel forms of induced cytochrome P-450 (*7*). This P-450-dependent monooxygenase activity is also widely distributed in fish; the kidney, intestine, gill, spleen, stomach lining, pancreas, and heart all have detectable AHH activity, although specific and total activities of liver predominate (*1*).

Results from previous studies make it apparent that cytochrome P-450-dependent metabolism in laboratory mammals and fish is similar; this is consistent with the idea that fish species will be suitable for testing the carcinogenic potential of certain types of chemicals. However, one must remember that 1) certain organs (e.g., lungs) are absent in fish, an important consideration for target organ carcinogenesis, and 2) chemokinetics can be markedly different in fish and mammals (fish oxidatively metabolize most xenobiotics more slowly than do warm-blooded animals). Thus a great deal of validation will be required for any fish species before it should be relied upon as a single in vivo test system for new chemicals. The fact that fish, such as rainbow trout (*Salmo gairdneri*), are sensitive to certain chemical carcinogens (*8*) demonstrates conclusively their potential as test animals and as environmental monitors for selective chemical pollutants. In the latter context, the presence of pollutant-mediated tumors or of specific biochemical changes might serve as a valid end point for a sentinel system.

Here we describe the heterogeneity of some monooxygenase (AHH and 7-ERD) activities in 2 feral marine teleost species of Maine, consider the possible association of elevated hepatic monooxygenase activity and exposure of the fish to PAH-type inducers present in their environment, and discuss some implications of variable monooxygenase activities in a single species in carcinogenesis testing and biochemical monitoring for selected pollutants of the marine environment.

MATERIALS AND METHODS

Chemicals.—All biochemical co-factors and buffers used were analytical grade. Benzo[*a*]pyrene was obtained from Sigma Chemical Company (St. Louis, Mo.); HCB and 4-CB (0.93 mCi/mmol) were both synthesized by the Laboratory of Environmental Chemistry, National Institute of Environmental Health Sciences.

Fish capture and tissue preparation.—Winter flounder (*Pseudopleuronectes americanus*) were caught by bottom trawl from the same area (4 miles seaward of Sand Beach, Mount Desert Island, Maine) and transferred to a 1,000-gallon tank equipped with flowing seawater (12–15° C). *Fundulus heteroclitus* were also captured consistently in the same area (Kings Creek on northwestern Mount Desert Island) with a seine net. The fish were held in 10-gallon aquaria equipped with flowing seawater. After a minimum acclimatization period of 2 days, the fish were decapitated. Livers (minus the gallbladders) were removed immediately and placed on ice in 1.15% potassium chloride, 1.25 m*M* HEPES, pH 7.4 (solution A), were blotted, weighed, and put in a Potter-Elvehjem homogenizer. Four passes of a motor-driven Teflon pestle were used. The concentration of the liver homogenates was 15% weight/volume.

When required, we prepared hepatic microsomes by first centrifuging the homogenate at 13,000×*g* for 20 minutes to remove cell debris, nuclei, and mitochondria. The resultant supernatant was ultracentrifuged at 176,000×*g* for 45 minutes; the microsomal pellet obtained was resuspended by hand in solution A and then centrifuged again at 176,000×*g* for 30 minutes.

Enzyme assays.—We assayed AHH activity essentially according to Wattenberg et al. (*9*). An NADPH-regenerating system was used that contained 5 m*M* each of glucose-6-phosphate (disodium salt) and magnesium sulfate, 0.5 m*M* NADP, 2 U of glucose-6-phosphate dehydrogenase, and 4 mg bovine serum albumin. The incubation mixture was buffered at pH 7.6 with 0.1 *M* HEPES and had a final volume of 2.5 ml. Incubation, usually for 20 minutes at 30° C, was started by the addition of the substrate (0.63 m*M* final added in 0.05 ml acetone), and the reaction was stopped by the addition of 1 ml cold acetone. Blanks were incubated under identical conditions, except that the substrate was added immediately after the addition of the acetone. Heptane (7 ml) was then added to the incubation mixture after which it was sealed, vortexed, and allowed to sit overnight in the dark at 4° C. The following day, after 2.5 ml of 1 *N* sodium hydroxide were added to a 2.5-ml aliquot of the heptane extract of each sample, the mixture was vortexed, centrifuged, and the fluorescence of the aqueous layer read on a Turner 430 fluorometer at an excitation of 400 nm and emission of 525 nm. Results are expressed in fluorescence units; 1 FU equals the fluorescence of a 3 μg/ml solution of quinine sulfate·$2H_2O$ in 0.1 *N* sulfuric acid at these same wavelengths. Either ANF or metyrapone was added to some incubation mixtures (0.5 m*M* in 0.05 ml acetone) immediately before the substrate. Based on 3 mg protein/incubation mixture, the minimum detectable AHH activity was 0.010 FU/minute per mg protein.

The method of Burke and Mayer (*10*) was followed for the assay of 7-ERD activity at 7-ethoxyresorufin and NADPH concentrations of 2 and 20 μM, respectively, and at a final pH of 7.8. The incubation mixture was internally standardized with resorufin. The minimum detectable level of enzyme activity with this substrate was 2 pmol resorufin formed/minute per mg protein.

To quantitate the formaldehyde formed, a measure of BND activity, we used a method based on that reported by

Cochin and Axelrod (*11*). An NADPH-regenerating system was used as described above for the AHH activity. Reaction mixtures that were incubated in parallel with the test reactions and to which a substrate (benzphetamine) was added after the reaction was stopped with zinc sulfate were used as blanks.

Cytochrome P-450 content of microsomes was quantitated by the dithionite difference procedure with an Aminco DW-2 spectrophotometer in the split beam mode (*12*). The protein content of various subcellular fractions was determined by the method of Lowry et al. (*13*) with bovine serum albumin as the standard.

Collection of urine from winter flounder.—Winter flounder weighing approximately 250 g were catheterized through the external urinary papillae with an umbilical catheter (3.5 French; Sherwood Medical Industries, Inc., St. Louis, Mo.). The fish were then placed in a wire mesh basket and suspended in a 10-gallon aquarium equipped with flowing seawater. (Winter flounder have been maintained in this manner for up to 7 days.) Ten to 16 hours after insertion of the catheter, the fish were given im or ip injections of [^{14}C]antipyrine (10 mg and 0.8 μCi in 0.25 ml saline) or 4-CB (5 mg and 10 μCi/kg in 0.25 ml water:ethanol:Emulphor 620, 1:1:3). In some experiments with [^{14}C]antipyrine, catheterized flounder were placed in a steel tray with aerated seawater (1 liter) sufficient to maintain gilling. At the conclusion of these experiments, the sea water in the tray was reduced in volume to about 10 ml under reduced pressure. The radioactive content of the solution was determined. An aliquot of the solution was spotted on silica gel thin-layer chromatography plates (Whatman LQ5D). The plates were developed in ethyl acetate:isopropanol:ammonium hydroxide, 45:35:20, vol/vol for differentiation of antipyrine from its metabolites. The developed plates were scraped, and the radioactivity of various zones of the thin-layer chromatography plates was determined by scintillation counting.

Slab SDS-PAGE was performed according to Laemmli and Favre (*14*) with 7.5% polyacrylamide gels and a 3% stacking gel. Standards used and their molecular weights were bovine serum albumin (68,000), ovalbumin (45,000), and carbonic anhydrase (32,000). Protein bands were stained in 0.115% Coomassie brilliant blue R-250 for at least 45 minutes before the unfixed stain was washed away in several changes of 25% aqueous ethanol:8% acetic acid. Samples (2-3 mg microsomal protein) were treated for 2 minutes at 90° C in a solution containing 10% glycerol, 3% SDS, 5% 2-mercaptoethanol, and 0.004% bromphenol blue as a tracking dye. Treatment of fish with inducing agents was by ip injection of either a single dose of 100 mg 5,6-BNF/kg in corn oil (13 mg/ml) or 3 doses, 24 hours apart, of 10 mg DBA/kg also in corn oil (8 mg/ml). The animals were killed either 9 or 11 days after the initial injection.

Analysis of flounder liver homogenates.—Various flounder liver homogenates were spiked with 1 μg fluoranthene/g liver, and the PAH, extracted according to Bligh and Dyer (*15*), were isolated from these extracts by sequential chromatography on LH-20 Sephadex (*16*), chromatography on Florisil (*17*), and liquid-liquid partitioning (*17*). Recoveries of added standards through these homogenization, extraction, clean-up, and chromatographic procedures were 80% for fluoranthene, benzo[*a*]pyrene, and DBA from cottonseed oil and rat liver homogenate. The samples (in isooctane) were concentrated and run under 2 conditions on a Spectra-Physics model 8000-B HPLC with UV (296-nm) and fluorescence (excitation 360 nm and emission over 418 nm) detectors in series and at a flow rate of 2 ml/minute. The samples were initially injected onto a reverse phase LiChrosorb RP-8 column starting with 70% vol/vol acetonitrile/water; after 15 minutes the acetonitrile concentration was changed 1.6%/minute up to 95%. The samples were also injected onto a direct phase Chromegabond Diamine column starting in 100% heptane. After 15 minutes, a gradient of methyl *t*-butyl ether was started at 0.66%/minute up to a final ether concentration of 10%. Both systems will resolve 2-5 ring aromatic hydrocarbons, whereas the latter procedure will also separate 6 ring isomers.

RESULTS

Variation in Rate of 4-Chlorobiphenyl Metabolism In Vivo

Several winter flounder with catheters in their urinary papillae were given im injections of 5 mg 4-CB/kg. As seen in table 1, the amount of radioactivity excreted in the urine over a 24-hour period and in the bile of these fish at the time they were killed varied markedly, i.e., from about 2 to 38% of the dose in urine and almost a 30-fold difference in the concentration of radioactivity in bile. The fish that showed low excretion of radioactivity in urine also had little radioactivity in bile, whereas those fish excreting substantial amounts of urinary radioactivity as metabolites also had higher concentrations of metabolites in their bile.

Variation of Hepatic Cytochrome P-450-Dependent Monooxygenase Activity In Vitro

Subsequent to these in vivo experiments, we determined hepatic monooxygenase activities of winter flounder in vitro. Initially, substrates efficiently metabolized by PAH-induced forms of cytochrome P-450 in mammalian and fish liver were studied; 7-ERD activity and AHH activity (in the presence and absence of ANF) were measured. The flavonoid ANF is known to inhibit AHH activity in hepatic microsomes from PAH-treated rats or little skates (*Raja erinacea*) and to enhance AHH activity in hepatic micro-

TABLE 1.—*Urinary and biliary excretion (24 hr) of radioactivity after an im injection of 4-CB into feral winter flounder*[a]

Fish wt, g	Percent dose in 24-hr urine	Disintegrations/min per μl bile
250	38.2	3,136
245	25.8	3,710
400	15.2	1,467
240	13.4	4,724
205	5.8	635
275	1.7	116

[a] An injection was 9.36 μCi and 5 mg 4-CB/kg in water:ethanol:Emulphor 620 (1:1:3) (Fouts JR, Foureman GL, Bend JR: Unpublished data).

TABLE 2.—*Comparison of AHH and 7-ERD activities in hepatic microsomes prepared from individual female winter flounder*[a]

Fish No.	AHH activity, FU/min per mg protein		7-ERD activity, pmol/min per mg protein
	Without ANF	With ANF	
1	0.55	3.36	<2
2	1.65	3.21	175
3	2.86	2.38	219
4	3.06	2.72	305
5	4.51	3.40	450
6	8.36	3.57	219
7	9.59	2.91	610
8	23.10	5.84	947

[a] Comparisons of AHH activities were done in the presence and absence of 5×10^{-4} M ANF.

somes from untreated rats and little skates (*7, 18*). The results from 1 such experiment are shown in table 2. Heterogeneity of both hepatic microsomal 7-ERD and AHH activities is marked. A dichotomous response to in vitro ANF was also noted; in fish having low AHH activities, ANF enhanced AHH activity, whereas in flounder with high AHH, ANF acted as an inhibitor. Moreover, the AHH and 7-ERD activities parallel each other.

Similar experiments also demonstrated that the relative AHH and 7-ERD activities and the in vitro effect of ANF on AHH activity were the same in whole liver homogenate and in microsomes isolated from these homogenates (data not shown). Consequently, subsequent experiments often used liver homogenate as the enzyme source.

Characteristics of Hepatic Monooxygenase Activity

We sampled the winter flounder population around Mount Desert Island, Maine, over 4 years to establish the recurrence of this variability and to characterize it further (table 3). The highest single AHH activity was observed in 1978, the highest single 7-ERD activity in 1980. In 1981, a sharp decline was noted in the mean 7-ERD activity recorded.

Progressing from season to season, the mean AHH activity was not significantly different ($P>0.05$) from that of the prior or following seasons. However, the mean hepatic AHH activities of 1979 were significantly different from those of 1981.

Annual variation was also considerable (49 to 84%) in the number of flounder with hepatic AHH activity inhibited by ANF (table 3). This parameter increased from 1978 through 1980, but a marked decrease from 84 to 68% was observed in 1981. Chi-square analysis of these percentages by season showed significant differences between 1978 and 1979 ($P<0.01$) and between 1980 and 1981 ($P<0.02$).

Comparison of Hepatic Microsomal Cytochrome P-450 Content and Aryl Hydrocarbon Hydroxylase Activity

With the exception of fish #1, the cytochrome P-450 content of flounder liver microsomes showed poor correlation with AHH activity, expressed per nanomole cytochrome P-450 (table 4). Also, no obvious relationship between the wavelength of maximum absorption of cytochrome P-450 and AHH activity was observed, although the maximum absorption of the fish with the highest AHH activity and the highest microsomal cytochrome P-450 concentration was shifted toward 448 nm, which is indicative of the presence of large amounts of cytochrome P-448 in these microsomes. No sex-related differences in the cytochrome P-450 content or AHH activity of the winter flounder were noted.

Comparison of Benzphetamine Demethylase and Aryl Hydrocarbon Hydroxylase Activities in Liver Homogenates

The activities of BND and AHH were determined in liver homogenates from several winter flounder (table 5), and no correlation was detected. The fish with the highest AHH activity had lower BND activity than did the animal with lowest AHH. The variation of AHH in these fish was wide (about 25-fold), whereas the variation in BND activities was just slightly more than twofold. We also found that 0.5 mM metyrapone, added in vitro, had little, if any, effect on hepatic AHH activities. These data are consistent with BND activity of flounder liver being catalyzed by non-PAH-inducible forms of cytochrome P-450 and with the elevated AHH activities found in many of the fish being catalyzed by PAH-inducible forms of the cytochrome.

TABLE 3.—*Summation of hepatic AHH and 7-ERD activities of feral Maine winter flounder over 4 yr*[a]

Date sampled	No.	AHH activity, FU/min per mg protein		7-ERD activity, pmol/min per mg protein		Percent fish showing inhibition of AHH activity by ANF
		Mean±SD	Range	Mean±SD	Range	
June–August 1978	172	1.78±1.66	0.04–8.80	131.3±128.0	<2– 837	49[b]
June–August 1979	81	2.03±1.88[c]	0.10–7.96	114.2±142.5	<2– 796	72[b]
June–August 1980	86	1.78±1.57	0.08–6.10	236.9±215.6	<2–1,165	84[d]
July–August 1981	71	1.31±1.23[c]	0.06–6.40	61.6± 55.4	<2– 196	68[d]

[a] Activities were determined in liver homogenate.

[b] Value was significantly different by chi-square analysis ($P<0.01$).

[c] Value was significantly different by Duncan Multiple Range Test ($P<0.05$; 2-tail test).

[d] Value was significantly different by chi-square analysis ($P<0.02$).

TABLE 4.—*Cytochrome P-450 content and AHH activity of hepatic microsomes from several feral winter flounder*

Fish No.	Cytochrome P-450 content, nmol/mg protein	Maximum absorption, nm	AHH activity, FU/nmol cytochrome P-450 per min
1[a]	0.60	448.4	6.4
2[b]	0.25	451.0	0.7
3[a]	0.23	450.4	1.1
4[a]	0.18	450.2	3.1
5[a]	0.16	450.8	1.1
6[a]	0.12	450.2	1.8
7[a]	0.12	450.0	0.9

[a] The AHH activity was inhibited by in vitro addition of 0.5 m*M* ANF.
[b] The AHH activity was increased by in vitro addition of 0.5 m*M* ANF.

Lack of Association of the Heterogeneity of Flounder Hepatic Aryl Hydrocarbon Hydroxylase Activity With Physical Characteristics of the Fish

We tested several physical parameters of the flounder to elucidate any correlation with hepatic AHH activity; weight and length, both indexes of age, and liver weight: body weight ratios were examined. Other parameters investigated included gonad weight, gonad weight:body weight ratio, sex, feeding status (presence or absence of particulate matter in the gut), and time (month) within the period from June to August. Absolute values of the correlation coefficients for the variables were low and ranged from 0.12 for gonad weight:body weight, to 0.29 for the liver weight:body weight ratios. The color of the livers, which is a crude estimate of their fat content, did not appear to correlate with specific or total hepatic AHH activity.

In Vivo Measurement of Cytochrome P-450-Dependent Monooxygenase Activity with [^{14}C]Antipyrine

Flounder with cannulated urinary papillae received im injections of 2 or 10 mg [^{14}C]antipyrine/fish. We found that a maximum of only 5% of the dose was excreted in the urine over a 24-hour period after treatment. Due to this low urinary excretion, we tried to determine if [^{14}C]antipyrine or some of its metabolites, or both, was eliminated by the gills. A catheterized flounder was placed in a stainless steel pan in seawater that was continuously aerated. The fish was treated im with 10 mg and 1 μCi [^{14}C]antipyrine. Forty-eight hours after administration of the drug, approximately 40% of the radiolabel was present in the seawater, whereas only 2% was present in the urine (fig. 1). Analysis by thin-layer chromatography demonstrated that all the radioactivity in the seawater was unchanged [^{14}C]antipyrine, which apparently had been excreted through the gills.

TABLE 5.—*BND and AHH activities in liver homogenate from several feral winter flounder*

Fish No.	BND activity, nmol/mg protein per min	AHH activity, FU/mg protein per min
1	0.60	0.2
2	0.47	1.3
3	0.58	1.9
4	0.36	4.5
5	0.42	5.2
6	0.78	5.8
7	0.53	6.8
8	0.46	7.3

Treatment With Polycyclic Aryl Hydrocarbon-Type Inducers and Comparison of the Hepatic Monooxygenase Systems in the Treated and Feral Flounder

After treating fish with either DBA or BNF, we observed that *all* fish had high 7-ERD activities (630–1,790 pmol/min per mg protein homogenate) and high AHH activities (3.0–13.0 FU/min per mg protein homogenate). In all treated flounder, hepatic AHH activity was inhibited by ANF (data not shown).

Hepatic microsomes were prepared from these fish pretreated with DBA and BNF, as well as from untreated flounder, and they were compared by SDS–PAGE. An electrophoretogram obtained from microsomes of 2 untreated flounder (channels 1 and 2), 1 BNF-treated flounder (channel 4), and 1 DBA-treated flounder (channel 5) is shown in figure 2. An intensely staining band of approximately 57,000 daltons was present in microsomes from treated flounder. A faint band of the same molecular weight is present in microsomes from both untreated fish. The presence and staining intensity of this 57,000-molecular weight band tend to correlate with the AHH activity of the liver homogenates from these fish (1.6, 0.7, 13.0, and 10.6 FU/min per mg protein for channels 1, 2, 4, and 5, respectively). Thus this electrophoretic band though present was faint in the untreated flounder with the lower AHH activities but was a major band in those fish that were pretreated with PAH-type inducers in which AHH activities were high. Similar data were obtained when hepatic microsomes from untreated winter flounder with divergent

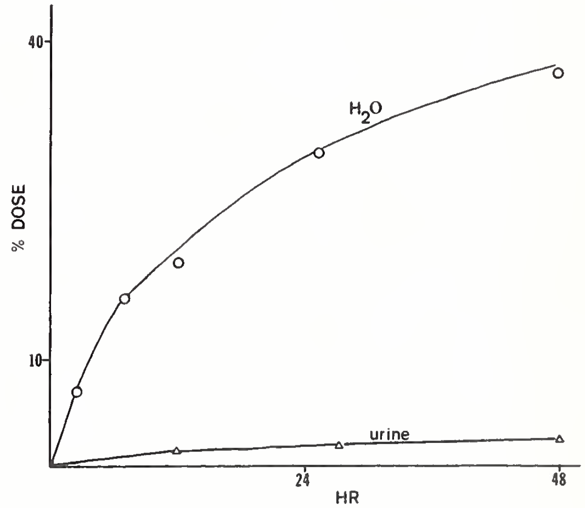

FIGURE 1.—Percentage of an im-injected dose of [^{14}C]antipyrine (1.0 μCi, 10 mg) excreted in the urine and into seawater by a winter flounder over 48 hr after treatment. Material in the sea water was unchanged antipyrine by thin-layer chromatographic analysis.

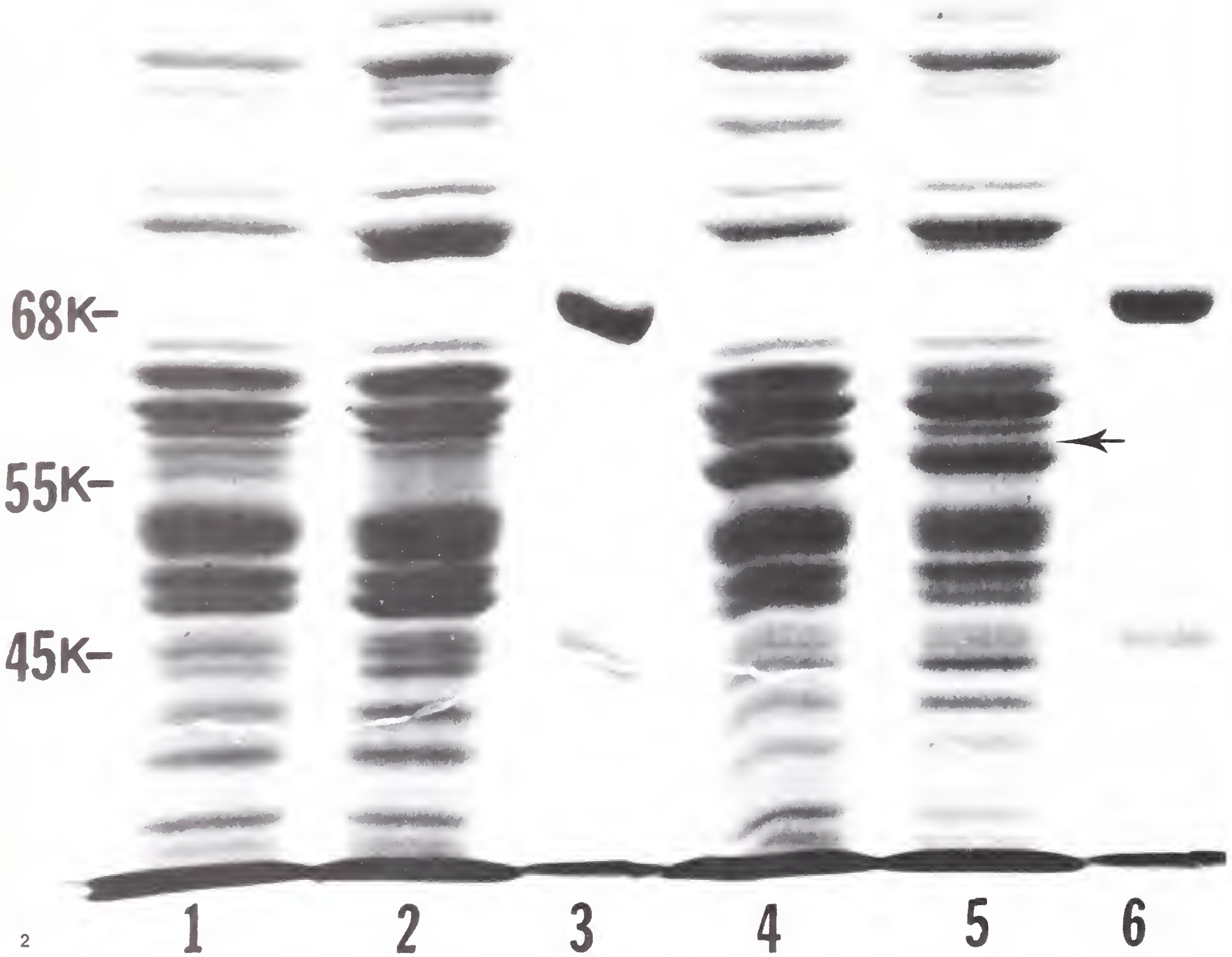

FIGURE 2.—Electrophoretogram of hepatic microsomes from 2 untreated flounder: a DBA-induced flounder treated ip with 10 mg DBA/kg on 3 consecutive days that was killed 8 days after the last dose, and a BNF-induced winter flounder treated ip with 100 mg BNF/kg that was killed 10 days later. Microsomal protein (225 μg) was applied to channels 1, 2, 4, and 5. Electrophoretic standards of bovine serum albumin (5 μg, 68,000 daltons) and ovalbumin (5 μg, 45,000 daltons) were applied to channels 3 and 6. The AHH activities expressed in fluorescence units/minute per milligram protein homogenate and the origin of the hepatic microsomes are as follows: channels 1 and 2, 1.6 and 0.7, untreated flounder; channel 4, 13.0, BNF-treated fish; and channel 5, 10.6, DBA-treated flounder.

AHH activities were subjected to SDS-PAGE, as shown in figure 3. Microsomes were electrophoresed in order of increasing AHH activity from left to right. The band of approximate molecular weight 57,000 (*see arrow*) became more prominent as the activity of the liver homogenates increased. This polypeptide band is probably an inducible form of cytochrome P-450 because it is readily apparent in a DBA-treated flounder (channel 3, fig. 2) with elevated AHH and 7-ERD activities but is barely visible or invisible in hepatic microsomes from a flounder with low AHH activity and no detectable 7-ERD activity (channel 1, fig. 4).

Analysis of Flounder Liver for the Presence of Polycyclic Aromatic Hydrocarbon Residues

Liver homogenates from 5 fish with high hepatic AHH activity (>5.8 FU/min per mg protein homogenate), from 3 fish with low hepatic AHH activity (<0.13 FU/min per mg), and from 3 flounder that had been treated with a total of 30 mg DBA/kg 7 days before death were individually extracted so that we could isolate any unmetabolized PAH present. The organic extracts obtained were analyzed by HPLC with an absorbance detector optimized for benzo[*a*]-pyrene and a fluorescence detector optimized for DBA.

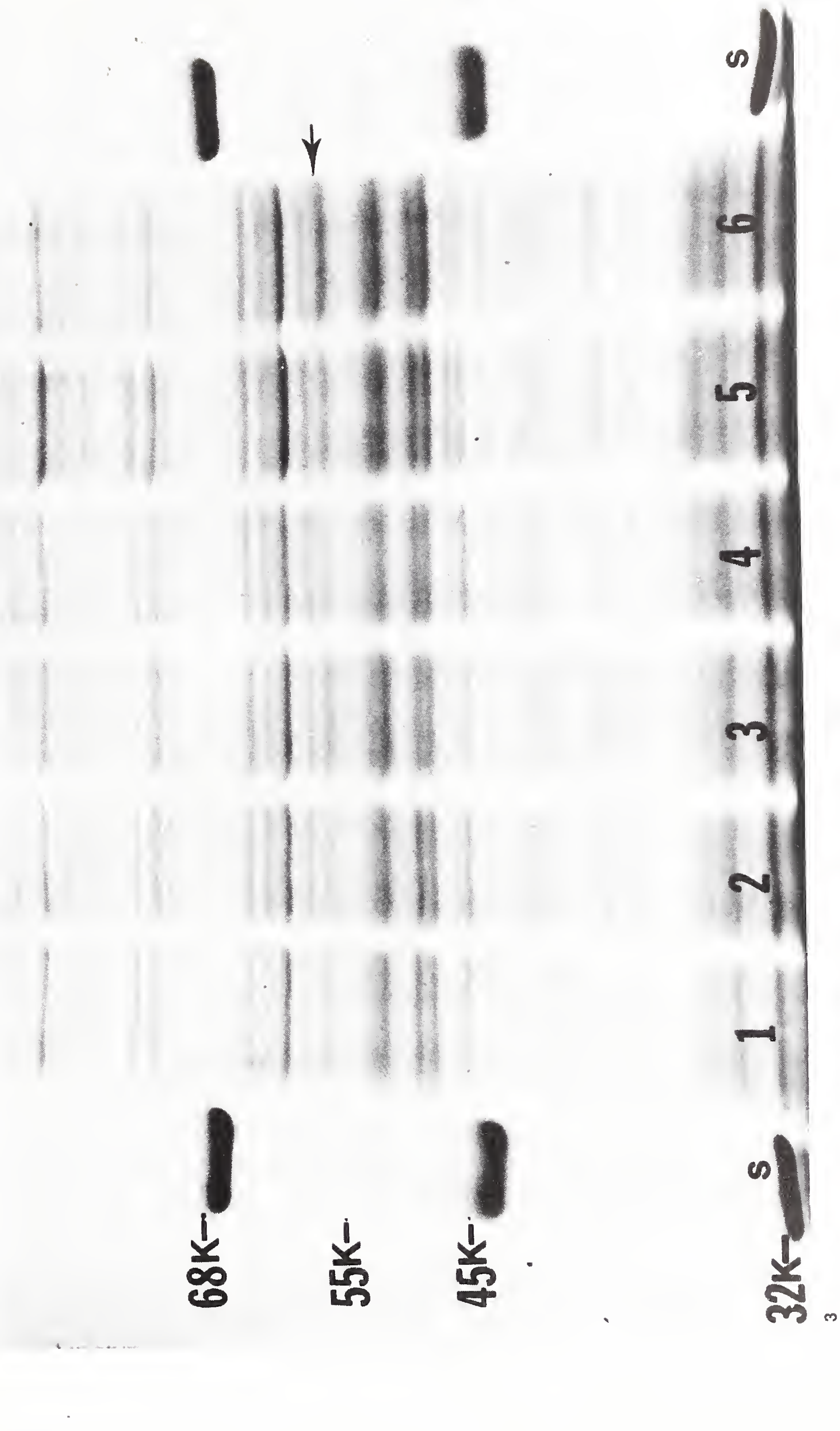

FIGURE 3.—Electrophoretogram of hepatic microsomes from feral winter flounder. Fifty micrograms microsomal protein from an individual fish were applied to channels 1 through 6. The AHH activities were 1.09, 1.66, 2.01, 3.45, 3.89, and 6.08 FU/min per mg protein homogenate for 1 through 6, respectively. Standards of 5 μg bovine serum albumin, ovalbumin, and carbonic anhydrase (32,000 daltons) were applied.

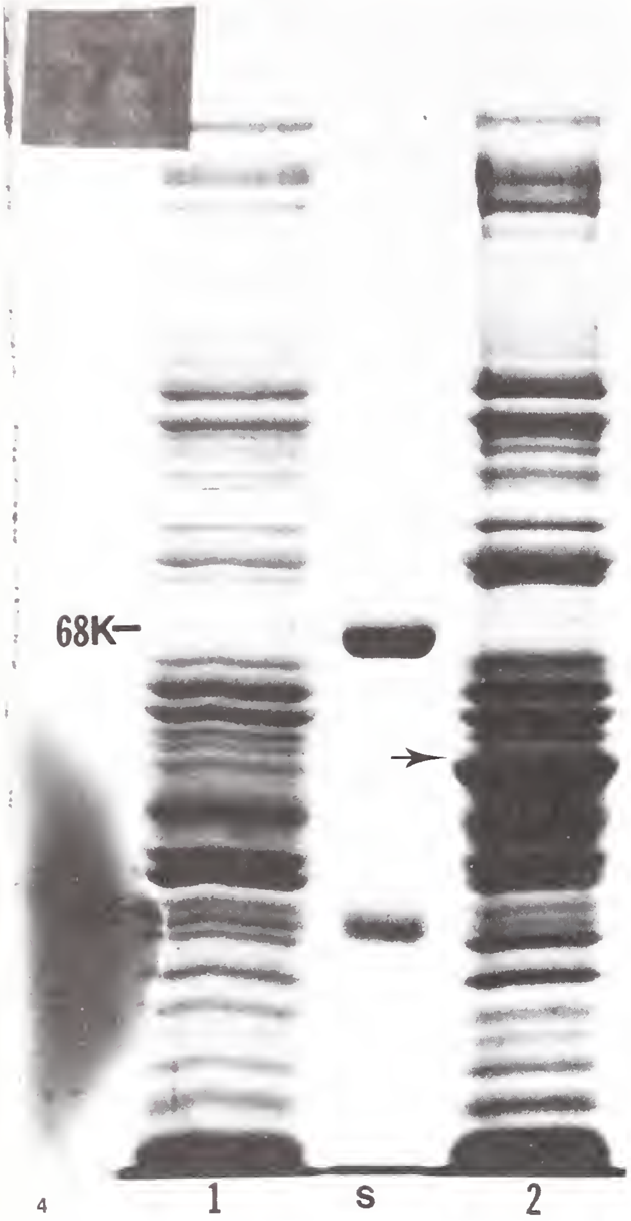

FIGURE 4.—Electrophoretogram of hepatic microsomes from an untreated (channel 1) and a DBA-induced (channel 2) winter flounder. *See* legend to figure 2 for additional information. The AHH activities were 0.31 and 4.00 FU/min/per mg protein homogenate, respectively. Untreated fish had no detectable 7-ERD activity (<2 pmol/min per mg protein).

Despite 0.5-μg detection capability for these compounds, neither was found in the liver homogenates from wild flounder with low or high AHH activities. Although the concentrations of unchanged hydrocarbon were too low to be accurately quantitated, DBA was present in the liver of all treated flounder. The amount of unchanged DBA in the liver of each fish represented less than 1% of the injected dose.

Characteristics of the Hepatic Monooxygenase System of Feral *F. heteroclitus*

To ensure that the wide variation of hepatic AHH and 7-ERD activities observed in winter flounder was not peculiar to this species, we performed a similar study with *Fundulus*. The AHH and 7-ERD activities of fresh hepatic homogenate from approximately 200 fish were examined during a single summer season. Once again, wide individual variations in both AHH activity (0.11 to 5.41 FU/min per mg protein homogenate) and 7-ERD activity (<2 to 244 pmol/min per mg protein) were observed and in vitro ANF inhibited AHH activity in most of the fish.

Experiments were also performed in an attempt at further induction of the hepatic monooxygenase system of *Fundulus* by the administration of HCB, which is a potent AHH-type inducer. Results obtained when hepatic AHH and 7-ERD activities were analyzed at various times after a single im injection of 25 mg HCB/kg are shown in table 6. The only significant increase in AHH activity was noted 6.5 days after treatment, although the mean AHH activity of the treated animals was slightly elevated in relationship to controls that received corn oil by injection in each instance. The 7-ERD activities of the HCB-treated fish were significantly greater than the controls at each point analyzed, which indicated that the feral *Fundulus* are only partially induced and that they are responsive to PAH-type compounds.

DISCUSSION

The large variation in the extent of urinary (22-fold) and biliary excretion of 4-CB metabolites by intact winter flounder (table 1) is consistent with the large variation in the rates of cytochrome P-450-dependent AHH (42-fold) and 7-ERD activities in the same species (table 2). As other studies have demonstrated that the rate of urinary excretion of polar compounds (*19*), including xenobiotic conjugates (*20*) by winter flounder in vivo, varies little, it seems clear that the cytochrome P-450-dependent reaction is the rate-limiting step in the urinary and biliary clearance of

TABLE 6.—*Effect of HCB administration on hepatic AHH and 7-ERD activities in female* F. heteroclitus[a]

Time after treatment, days	AHH activity, FU/min per mg protein homogenate		7-ERD activity pmol/min per mg protein homogenate	
	Control	Treated	Control	Treated
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
3.5	2.7±0.6	3.4±1.1	76±32	138±39[b]
6.5	2.0±1.0	5.0±1.6[c]	114±67	205±34[b]
10.5	1.5±0.8	1.6±0.1	124±75	194±37[b]

[a] Fish received im injections of 25 mg HCB/kg in corn oil. Control fish were treated with corn oil only. Values for 4 fish were calculated in the 3.5-day groups, and for 5 in the 6.5- and 10.5-day groups.

[b] Values were different from paired controls, $P < 0.05$.

[c] Values were different from paired controls, $P < 0.01$.

4-CB metabolites. This will also be true for many procarcinogens in fish and may contribute to whether test chemicals lead to carcinogenesis in an individual fish. In the simplest situation, when a single ultimate carcinogen (electrophilic metabolite) is formed from the procarcinogen, either extreme of rapid or slow metabolism could favor alkylation of DNA, the presumed initiation step in chemical carcinogenesis. If the target is the liver hepatocyte, more rapid metabolism by this cell might overwhelm any detoxication pathways present, such as conjugation with the nucleophile glutathione, and DNA-adduct formation would increase. If hepatic metabolism is slow, procarcinogen concentrations in extrahepatic tissues and cells will tend to be higher for prolonged periods. For example, if the cells susceptible to carcinogenesis are in the kidney or gill, slower procarcinogen metabolism in the liver may enhance DNA alkylation in the extrahepatic cells with greater risk for the expression of carcinogenesis. Thus a case can be made for the inclusion of individuals with varying rates of hepatic monooxygenation in an in vivo experiment in which chemicals are being tested for carcinogenic potential and the site of tumorigenesis is unknown.

The marked variation noted in hepatic AHH and 7-ERD activities (table 2) prompted us to investigate this phenomenon in more detail. Over a 4-year period, at least 49% and as many as 84% of the flounder sampled had hepatic AHH activities that were elevated and were inhibited by in vitro ANF (table 3). By analogy to our earlier studies with marine fish (*7*) and the studies of others with mammals (*18, 21*), this inhibition of AHH activity suggested the presence of a novel form or forms of cytochrome P-450 similar to or identical in biochemical characteristics to forms of cytochrome P-450 induced by treatment with PAH-type compounds. These chemicals include many PAH, certain polychlorinated biphenyl isomers (e.g., HCB), certain polybrominated biphenyl isomers, halogenated dibenzo-*p*-dioxins, halogenated dibenzofurans, certain flavonoids (e.g., BNF), and isosafrole, and they represent compounds of both natural and anthropogenic origin.

Further experiments supported this concept. The flounder with the highest AHH activity whose cytochrome P-450 content was measured (table 4, fish 1) had twice as much cytochrome P-450 (0.60 nmol/mg microsomal protein) as any other fish sampled. The maximum absorption of the carbon monoxide-ligated and reduced cytochrome P-450 spectrum of this fish also showed a marked hypsochromic shift (448.4 nm) relative to the fish with the lowest AHH activity (451.0 nm). All the other flounder listed in table 4 with AHH activity inhibited by in vitro ANF contained cytochrome P-450 with a maximum absorption band that occurred between 450.0 and 450.8 nm. Thus a hypsochromic shift, although sometimes minor, was observed in the cytochrome P-450 spectrum of each fish with ANF-inhibited AHH activity. Similar observations have been made in marine fish treated with PAH, in species in which no feral fish have high AHH activity inhibited by ANF. Large increases in hepatic microsomal AHH and 7-ERD activities are known to occur in the absence of increases in specific cytochrome P-450 content or of any hypsochromic shift in the cytochrome P-450 spectrum (*7*); in other instances, increases in cytochrome P-450 content and a hypsochromic shift in the hepatic microsomal cytochrome P-450 spectrum accompany induction of AHH and 7-ERD activities in fish (*22*). For these reasons it becomes obvious that neither microsomal cytochrome P-450 content nor the maximum absorption of the P-450 spectrum serves as a valid parameter in fish for those considering a biochemical end point for detecting the occurrence of PAH-type inducing agents as environmental pollutants.

From investigations with individual forms of cytochrome P-450 isolated from liver of various mammals, we know that considerable substrate selectivity occurs, although overlap does as well (*23–26*). Benzphetamine is a compound that is usually not a substrate for PAH-induced forms of cytochrome P-450, and so BND activity was compared with that of AHH in the livers of several flounder. The fact that AHH activities varied more than 30-fold, whereas those of BND varied only about twofold (table 5), is consistent with much of the AHH in fish with high activity being catalyzed by 1 or more forms of cytochrome P-450 induced by PAH-type chemicals and that do not demethylate benzphetamine effectively. This was verified by SDS–PAGE (figs. 2–4). A major electrophoretic band with a molecular weight of approximately 57,000 was present in hepatic microsomes from winter flounder treated with either DBA or BNF (figs. 2, 4). This band was minor in hepatic microsomes from feral flounder that had low AHH activity (figs. 3, 4) but became more pronounced as hepatic AHH activities increased. We emphasize that equivalent amounts of protein were loaded into each of the gels, and comparisons in intensity were made after protein staining. Although only this single major band could be associated with the increased AHH and 7-ERD activities in the electrophoretograms, probably at least 1 other minor PAH-induced form of cytochrome P-450 is also involved in the AHH and 7-ERD heterogeneity because these activities did not change concomitantly from year to year (table 3). For example, from 1980 to 1981, hepatic 7-ERD activity decreased 385%, but AHH activity decreased only 135%.

The experiments performed with *F. heteroclitus* (table 6) demonstrated conclusively that the variability of 7-ERD and AHH activities and the in vitro inhibition of AHH activity by ANF in certain fish were not characteristic only of feral winter flounder; they occurred also in another Maine marine teleost.

The presence of PAH-type inducers as residues in liver of winter flounder with high AHH activity would support the assertion that these elevated enzyme activities were due to exposure to chemicals of this type. However, one must remember that fish metabolize many PAH-type compounds in vivo and excrete them (*27*), and the more highly induced the monooxygenase system is, the faster this metabolism should be. Consequently, it is really not surprising that we failed to detect any PAH in feral flounder with high AHH activity, especially because less than 1% of the administered DBA (30 mg/kg) was present in flounder liver about 1 week after its parenteral administration. What is obvious is that our analyses cannot verify any role for environmental PAH in the high AHH activities of many feral flounder. The data also stress the fact that monitoring fish liver and probably other tissues as well for unchanged PAH as an index of environmental pollution is not a wise course to follow; stationary marine invertebrates metabolize xenobiotics much more slowly and are a much better choice for this sort of chemical vigilance.

The causative factor for the large variation in hepatic AHH and 7-ERD activities in the winter flounder could be genetic as well as environmental. Other explanations seem to be precluded because no biologically significant correlation occurred among any of the physical characteristics studied (fish length, body weight, liver weight, liver weight:body weight, sex, gonad weight, gonad weight:body weight, month, feeding, or starvation) and hepatic AHH activity. The possibility that the observed variation in some enzyme activities may have as its basis an *Ah* locus analogous to that present in mice (*28*) seems unlikely because *all* the winter flounder treated with PAH showed induction of AHH and 7-ERD activities. Thus an environmental explanation seems preferable. Flounder are demersal fish and spend nearly their entire lives burrowing into sediment (*29*). Such bottom sediments are generally recognized as an important repository for various petroleum-derived pollutants (*30, 31*), and they often contain appreciable amounts of PAH (*32*). Published data also demonstrate the accumulation of lipophilic xenobiotics from sediment by flatfish (*33, 34*) and support the sediment-to-teleost link for pollutant transport within the marine food web. Environmental factors may also account for the *Fundulus* data. These fish are free-swimming but they generally remain close to shore (within 30 meters) in muddy pools, ditches, or creeks (*35*). *Fundulus* can survive in foul water and have ample opportunity for exposure to PAH-type pollutants and other PAH inducers of natural origin, such as plant-derived flavones (*36*). The flounder with the highest AHH activities are probably more sensitive to PAH-type enzyme inducers (i.e., show more induction of the P-450-dependent monooxygenase system at similar inducer concentrations) because the variations in AHH and 7-ERD activities were noted over 4 seasons in fish that were captured from the same area off Mount Desert Island, Maine. Migration of flounder exposed elsewhere to different local amounts of pollution to Mount Desert Island may also be a factor here but it is significant, in this context, that winter flounder are considered to be one of the least migratory marine fish in North America, as they are comprised of local independent populations (*36*).

Because winter flounder are excellent in vivo fish models (*37*) for studying the renal handling of polar compounds, including xenobiotic metabolites, it would be advantageous if investigators could assess the status of their hepatic monooxygenase system before conducting similar studies with chemicals that are substrates for PAH-induced forms of cytochrome P-450. Antipyrine has been successfully used in laboratory animals (*38*) and in humans (*39*) for this purpose. Our study with flounder (fig. 1) showed this compound to be inappropriate for the in vivo assessment of monooxygenation because it was effectively excreted unchanged by the gills.

That 1) multiple forms of cytochrome P-450 are present in liver and presumably in other extrahepatic tissues of fish, 2) some forms of P-450 can be induced by PAH-type chemicals present in the environment, and 3) quantitative differences may occur in the response of the hepatic monooxygenase system to PAH-type chemicals in the aquatic environment all have relevance to testing for carcinogenesis is well-known. Different forms of cytochrome P-450 show substrate selectivity, including both regioselectivity and stereoselectivity (*40*) that can be highly important in the conversion of a procarcinogen to an ultimate carcinogen. The PAH-type enzyme induction decreases the amount of specific DNA adducts formed from [^{3}H]benzo[*a*]pyrene in cells of the target organ (e.g., lung) and of the incidence of tumors in mice (*41, 42*). Similar effects are to be anticipated in fish. Considerations such as these suggest that extensive study is required for the validation of any fish species for carcinogenesis testing. Ideally, attention should be given to dose–response relationships, to the comparison of specific DNA-alkylation products in responsive and nonresponsive cells or tissues with the incidence of tumorigenesis, to thorough characterization of metabolic systems in hepatic and extrahepatic tissues, to the toxicokinetics of various procarcinogens in these fish, and to DNA repair processes in susceptible tissues or cells of a fish species. Such background information will be necessary if we are to interpret accurately the data on the comparative tumorigenesis of carcinogens of different chemical classes in fish and in mammals; it is also important for the validation of carcinogenesis testing in fish. The information available to date suggests that fish have considerable potential for use in carcinogenesis testing systems, primarily because of economic factors. It is the responsibility of investigators working in this area to validate this approach thoroughly before such testing becomes widely accepted by the scientific community.

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DISCUSSION

K. Hoover: Dr. Bend, have you tested the enzyme levels in fish elsewhere, i.e., gone to the South Pacific or somewhere in a pristine area?

J. R. Bend: No, we have not. In fact, one of the reasons that we are doing this is to try species that dwell near shore to see, in fact, if there has been any opportunity for interaction with pollutants. I think Dr. Stegeman might talk a little bit about exotic species that have been dredged up from 2,000 feet below the ocean. The interesting point is the fact that he finds this sort of enzyme induction in some of these species from midocean, 3,000 feet down.

J. Stegeman: That is 3,000 meters.

M. Landolt: Dr. Bend, did you investigate the feeding status of the fish?

Bend: Yes. I knew there was something I forgot to mention. What Dr. Foureman did was to open the intestines of large numbers of fish, and we gauged by the contents whether they were actively feeding. We saw no correlation between AHH activity and the presence or absence of food in the intestine.

Landolt: We noticed when we were working with the flat fish that those animals that consistently came up low were the ones that consistently were not feeding, and when we repeated the work with trout, we had the same results. When we observed high levels, it was always in the fish with food in their intestines, and when we saw low levels, they were in fish that for whatever reason were not actively feeding.

Bend: We definitely had fish that had been feeding that still had low AHH activities.

J. J. Lech: Dr. Bend, I know that you work with lung and also with mammalian tissues. Why do you think there is a tissue difference or dose relationship between benzo[*a*]pyrene-induced tumors, e.g., liver versus lung in the mammal? Why do we need such a high dose in the trout?

Bend: There is one answer. I think I will just talk about DNA alkylation and then the disappearance of specific adducts. Dr. Marshall Anderson is doing some work on in vivo benzo[*a*]pyrene. The idea behind the work was to study those tissues that are susceptible to carcinogenesis and those that are resistant. The tissues he is investigating in AHe/J mice are forestomach, lung, and liver. If you look at the amount of alkylation of guanine by benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide-1, you see almost the same level of alkylation in the forestomach and lung versus liver. If you time the disappearance, then you find that in lung the disappearance of the adduct is similar to the turnover of the epithelial cells. However, there is repair of the DNA in liver.

So what we are doing now is something that we have been doing with monooxygenase activity for some time, and that is getting into the question of cellular heterogeneity. We have methods in the laboratory for studying Clara cells, alveolar type II cells, and alveolar macrophages isolated from rabbit lung. We are also hoping to study endothelial cells because of the potential for involvement of the prostaglandin cooxygenase system, but we will be pretreating rabbits with radiolabeled benzo[*a*]pyrene of high specific activity, then isolating the cells and determining the amount of alkylation and repair by killing the animals and isolating cells at various times.

J. Couch: Dr. Bend, I was wondering, due to the unpredictable variability in the activity found in a lot of different organisms in different places, if this puts to rest, at least temporarily, the possibility of using this system as a monitoring tool for pollutant insult?

Bend: I think that is an important point. The approach that we were going to use was the test for specific cytochrome P-450 isozymes by inhibition of selected monooxygenase activities by monospecific antibodies. However, because the same forms of P-450 are induced by PAH-type inducers of anthropogenic and natural origin, there is a problem. Thus it is possible to monitor effectively a biological response but, without detailed chemical characterization of water and sediment, pollution cannot be implicated as the causative agent.

C. J. Dawe: Well, it seems to me that we could double the usefulness of some of the information we get from different disciplines and different sources, if they could be coordinated. For example, I mean we not only have tumors in liver of the tom cod in the Hudson River (as they say "down by the liver side"), we know that you have these variations in the metabolites. However, if the studies could be done in the same areas and then compared with the places where the tumors are occurring, I think the results would have a lot more significance.

Bend: I think that is an excellent point. One of the problems is that the experiments we do have to be done with fresh tissue. We have determined that activity is lost if tissue is frozen. Thus we conducted our studies at a well-equipped marine laboratory. However, we hope the same approach will be used by other people. We have specifically designed this biochemical test system so that it can be used with 100 μl of a liver homogenate, and the assays are straightforward. I hope that other people will apply these procedures to fish populations that show high liver tumor incidence.

G. Streisinger: You mentioned earlier that you would comment on measuring adducts at the DNA level. Have you actually done that and have you considered measuring adducts at the level of hemoglobin, as Dr. Lars Ehrenberg suggested, which may be easier to tackle?

Bend: Again, in mammalian systems we are measuring adducts at the DNA level. We are not studying alkylation of hemoglobin by chemical carcinogens in vivo in either mammals or fish.

Patterns of Benzo[*a*]pyrene Metabolism by Varied Species, Organs, and Developmental Stages of Fish[1, 2, 3]

John J. Stegeman, Bruce R. Woodin, *and* Robert L. Binder[4, 5]

ABSTRACT—Microsomal preparations of liver from adults of several (teleost) fish species, including scup (*Stenotomus chrysops*), winter flounder (*Pseudopleuronectes americanus*), and killifish (*Fundulus heteroclitus*), metabolized the model polycyclic hydrocarbon carcinogen benzo[*a*]pyrene (BP) with a marked regiospecificity, forming high percentages of benzo-ring derivatives, but particularly BP-7,8-dihydrodiol and BP-9,10-dihydrodiol. The identity of these products was established by mass spectrometry of metabolites formed by scup (*S. chrysops*) liver microsomes compared with the spectra obtained for authentic *trans*-dihydrodiols. Whereas the benzo-ring dihydrodiols equaled 40–60% of the total ethyl acetate-soluble metabolites formed by liver microsomes of the various species, the K-region metabolite BP-4,5-dihydrodiol was formed in small or undetectable amounts. Microsomal preparations of kidney and gill from *S. chrysops* formed BP-7,8-dihydrodiol and BP-9,10-dihydrodiol in proportions like those seen with liver of the same species. Embryonic tissues of *F. heteroclitus* metabolized BP with a regiospecificity similar to that exhibited by adult liver microsomes. Isomeric forms of BP-7,8-dihydrodiol are potent proximate carcinogens, and activation of BP to mutagenic and carcinogenic derivatives is associated with metabolism on the benzo ring of BP. The widespread appearance of regiospecificity for this portion of the BP molecule may be reflected in structural requirements for metabolism of other hydrocarbons and indicate that fish are useful in the study of various factors that affect tumorigenesis by polycyclic hydrocarbons. — Natl Cancer Inst Monogr 65: 371–377, 1984.

Strong evidence links the action of many chemical carcinogens to their metabolism and subsequent binding of reactive metabolites to cellular macromolecules, particularly to DNA (*1*). Among these carcinogens are the PAH, of which BP, a key example, is oxidatively transformed by cytochrome P-450-dependent monooxygenases to a number of epoxides. These epoxides are further transformed to dihydrodiols by epoxide hydrolase and to phenols and quinones by chemical transformation [*see* (*2*)]. Studies of the mutagenic and carcinogenic potential of BP and its metabolites indicate that an isomer of the "bay region" diol-epoxide 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene is the most potent mutagenic and carcinogenic form of BP (*3–6*). The isomeric benzo-ring diol-epoxides result from further metabolism of (±)-*trans*-7,8-dihydroxy 7,8-dihydrobenzo[*a*]pyrene (BP-7,8-dihydrodiol), and this dihydrodiol is accordingly the most potent proximate mutagenic (*7*) and carcinogenic (*8*) form of BP.

If metabolism at the benzo ring of BP is an important prerequisite for the carcinogenicity of this compound in many systems, as it seems to be, then its metabolism could be an important factor in BP tumorigenesis in a given species or organ. As with other such carcinogens, knowledge of the patterns of metabolism of BP is essential to the interpretation of tumorigenesis studies. Differences in the relative capacity to form benzo-ring metabolites of BP, particularly BP-7,8-dihydrodiol, could suggest differences in susceptibility to tumorigenesis. Moreover, the metabolite profiles of compounds, such as BP, may be useful in comparative studies of the catalytic function of cytochromes P-450 responsible for that metabolism. From studies with mammals, we know that various forms of cytochrome P-450 can possess different specificities for sites on the BP molecule, i.e., regiospecificity (*9–11*).

Previous studies have indicated that hepatic preparations of some (teleost) fish species efficiently catalyze the formation of benzo-ring dihydrodiols of BP (*12–14*). In this report, we confirm the identity of these metabolites produced by the marine fish scup (*Stenotomus chrysops*) and describe some features related to formation of such metabolites in vitro. We further disclose the general nature of this metabolic capacity in marine fish by examining profiles of BP metabolites produced by several species, by some extrahepatic tissues of fish, and by fish embryos. Several implications of these results are discussed.

MATERIALS AND METHODS

Chemicals.—We obtained the compounds used in our research from various sources, e.g., benzo[*a*]pyrene (Gold Label) from Aldrich Chemical Company, Milwaukee, Wisconsin; NADPH from Sigma Chemical Co., St. Louis,

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ABBREVIATIONS: PAH = polycyclic aromatic hydrocarbon(s); BP = benzo[*a*]pyrene; HPLC = high-pressure liquid chromatography; PCB = polychlorinated biphenyl(s); OH = hydroxy.

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[5] We thank Dr. Nelson Frew for his assistance with the mass spectrometry, and the staff of the Chemical Carcinogen Reference Standard Repository of the Division of Cancer Cause and Prevention, National Cancer Institute, for the benzo[*a*]pyrene metabolite standards.

Missouri; and solvents for various extractions and for HPLC from Burdick and Jackson, Muskegon, Michigan. Generally, [^{3}H]-labeled BP was obtained from Amersham-Searle, Skokie, Illinois, and Aquasol and [^{3}H]toluene from New England Nuclear, Boston, Massachusetts. Authentic metabolites of BP were obtained from the Research Institute of the Illinois Institute of Technology, Chicago, Illinois, through the courtesy of the National Cancer Institute Carcinogenesis Research Program. The PCB was Aroclor 1254 (lot AA-1) from the Monsanto Chemical Company, St. Louis, Missouri.

Animals.—We caught the scup (*Stenotomus chrysops*), ranging from 150–600 g, during September 1980, from local Woods Hole waters. Winter flounder (*Pseudopleuronectes americanus,* 500 g) were obtained by otter trawl in Buzzards Bay, Massachusetts. Tissues of both species were prepared within days of capture. Mummichog (*Fundulus heteroclitus*) ranging from 5 to 7 g were collected by seine at Herring Brook Marsh, North Falmouth, Massachusetts, and were maintained in the laboratory for several months before use. These animals were fed a diet of Purina Trout Chow from Ralston Purina Company, St. Louis, Missouri, and of smelt. Both *P. americanus* and *F. heteroclitus* were gonadally mature at the time of use. We obtained embryos of the latter species by stripping gametes and effecting fertilization in the laboratory. The fertilized eggs were maintained and *F. heteroclitus* embryos were treated with PCB as in our previous experiments (*15*). Adult fish of each species received no experimental treatment.

Tissue preparation.—All procedures were conducted at about 2–4° C. Livers of scup, winter flounder, and adult mummichog were excised, and microsomes were prepared from individual or pooled livers according to procedures and with materials as described (*16*). Scup kidney and gill tissues were removed from 23 animals, pooled appropriately, and microsomes were prepared as described (*16*). Microsomes were prepared from whole eleutheroembryos of *F. heteroclitus* after a pool of embryos that had been treated with PCB was homogenized. Modified centrifugal procedures yielded embryonic microsomes with characteristics like those obtained by standard procedures (unpublished results). Microsomal pellets from each preparation were resuspended in a buffer containing 50 m*M* Tris–HCl (pH 7.4), 1 m*M* EDTA, 1 m*M* dithiothreitol, and 20% glycerol by volume.

Preparation of metabolites.—All procedures were performed in the dark or under red light. Metabolites of either unlabeled of [^{3}H]BP were obtained by in vitro incubation with microsomes prepared from the various species and tissues. Metabolites were always prepared in 50 m*M* Tris–HCl buffer (pH 7.4–7.5) with BP added in acetone to a final concentration of 62 μM. Reactions were initiated by the addition of NADPH to a final concentration of 0.5 m*M*. Final reaction volume varied between 0.1 and 1.2 ml depending on the sample, and temperature was either 25° or 30° C, according to the optima determined for each species. Duration of the reactions varied between 10 and 30 minutes, depending on the activity of the sample and the amount of protein available. All reactions were stopped by the addition of a volume of ice-cold acetone equal to the reaction mixture volume. This mixture was subsequently extracted twice with volumes of ethyl acetate equal to twice the original reaction volume. The ethyl acetate extract was dried with hexane-washed magnesium sulfate, and evaporated under high purity nitrogen. Dried extracts were resuspended in 50–100 μl acetonitrile and held under nitrogen until analysis.

Metabolite analysis.—Metabolites dissolved in acetonitrile were separated by HPLC with the use of a DuPont 850 chromatograph fitted with a 50-μl injection loop, a 25-cm DuPont Zorbax ODS column, and a fixed wavelength detector. Metabolites were eluted by our running a gradient of acetonitrile and water (*17*). Amounts of material in peaks were determined by integration of peak areas with a Hewlett-Packard 3390 Integrator, by comparison with areas produced by known amounts of authentic standards, and by liquid scintillation counting when [^{3}H]BP was used as the substrate. The labeled peaks were collected, taken to dryness, resuspended in neutralized Aquasol, and determined to 1% error in a Beckman LS-100C liquid scintillation counter. Measured recovery of individual metabolites carried through the procedure averaged about 70%, and the data have been corrected for this.

Those metabolites to be analyzed by mass spectrometry were collected from the HPLC in chloroform-washed glassware, extracted twice with 1.5 ml chloroform, and dried under nitrogen. These fractions were resuspended in methylene chloride and subjected by probe distillation to electron impact ionization mass spectrometry on a Finnigan Model 1015. Crystals of authentic standards were dissolved directly in methylene chloride and similarly analyzed.

RESULTS

Analysis of BP metabolites formed in vitro by scup liver microsomes revealed several products (*13*) that could be identified on the basis of coelution with authentic standards (fig. 1A). Thus scup liver preparations in vitro form products eluting with BP-9,10-dihydrodiol, BP-7,8-dihydrodiol, BP-1,6-quinone, BP-3,6-quinone, 9-OH-BP and 3-OH-BP, the latter peak including 1- and 7-OH-BP as well (*17*). However, these same microsomal preparations did not form any BP-4,5-dihydrodiol or BP-6,12-quinone in amounts that could be seen at the limits of detection under the conditions of the assay used here (<0.1 pmol of product produced/min). The elution of authentic BP-4,5-dihydrodiol and BP-6,12-quinone relative to the metabolically produced derivatives is described in figure 1B.

Because formation of benzo-ring metabolites, particularly BP-7,8-dihydrodiol, is associated with formation of mutagenic derivatives, it is important that the identity of these products be unambiguous. Epoxide hydrolase activity is required for the production of the putative BP-9,10-dihydrodiol and BP-7,8-dihydrodiol by scup liver microsomes (*18*), which clearly indicates that they were dihydrodiol derivatives of BP. The identities of these as BP-9,10-dihydrodiol and BP-7,8-dihydrodiol were confirmed by mass spectral analysis of the metabolites produced in vitro (table 1). The molecular weight of each was

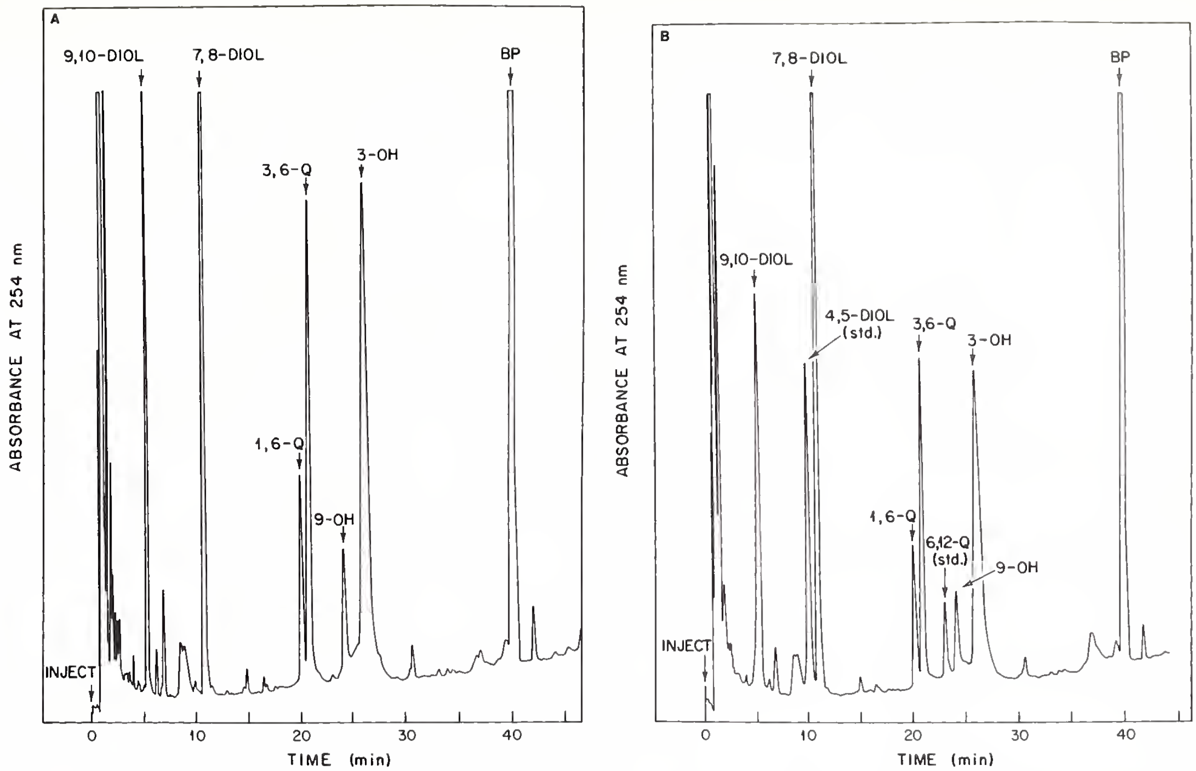

FIGURE 1.—A) HPLC analysis of BP metabolites formed by scup (*S. chrysops*) hepatic microsomes. Metabolites were eluted with gradient from 40 to 85% acetonitrile in water and detected by UV absorption at 254 nm. The microsomes were prepared from liver pooled from 20 fish and incubated at a concentration of 1.13 mg/ml for 15 min. The 3-OH-BP peak contains 1-OH-BP, 3-OH-BP, and 7-OH-BP, as described in (*17*). Unlabeled peaks in the vicinity of the dihydrodiols are putative secondary metabolites. B) An aliquot of the same metabolite preparation was injected at the same time as authentic BP-4,5-dihydrodiol and BP-6,12-quinone, both of which are designated as "std."

286, and the fragmentation patterns were virtually identical to those obtained for the corresponding authentic *trans*-dihydrodiols.

The rate of formation of the two benzo-ring dihydrodiols and of the phenol derivatives eluting with 3-OH-BP by scup liver increased linearly with increases in the protein concentration of reaction mixtures up to about 2.0 mg microsomal protein/ml (fig. 2). Beyond this, no further increase in the rate of production of these metabolites occurred. These latter 2 benzo-ring dihydrodiols accounted for more than 50% of the total ethyl acetate-soluble metabolites over most of the range of protein concentrations. In contrast, 9-OH-BP showed an initial high rate of formation that fell to negligible levels at 1.0 mg protein/ml, and this rate remained low at higher concentrations of protein as well; the reason for this has not been established. The rate of formation of quinone derivatives varied little over the range of protein concentrations nor was there any apparent formation of BP-4,5-dihydrodiol or of BP-6,12-quinone at any of the protein concentrations used.

TABLE 1.—*Mass fragments of authentic BP dihydrodiols and metabolites formed by scup liver microsomes*[a]

BP-7,8-dihydrodiol		Metabolite		BP-9,10-dihydrodiol		Metabolite	
m/z	Relative intensity	m/z	Relative intensity	m/z	Relative intensity	m/z	Relative intensity
286	46	*286*	53	*286*	78	*286*	76
268	51	268	35	268	56	268	72
240	84	240	90	242	70	242	70
239	100	239	100	239	100	239	100
226	27	226	29	226	32	226	33
215	21	215	24	214	59	214	56

[a] Metabolites were obtained from incubations described in the legend to figure 2. A selection of major fragments is listed. m/z = mass divided by charge.

As the protein concentration of reaction mixtures was increased, the appearance of peaks that did not elute with known compounds also increased. Many of these are presumed to be secondary metabolites resulting from further action of cytochrome P-450 on the dihydrodiol and phenolic derivatives of BP. The formation of 2 such products as a function of protein concentration is depicted in figure 3. Although their identity has to be established, the elution of these 2 peaks before that of BP-9,10-dihydrodiol is consistent with a greater polarity and a possible identity of these as either triols or tetraols.

The capacity for metabolism of PAH by extrahepatic

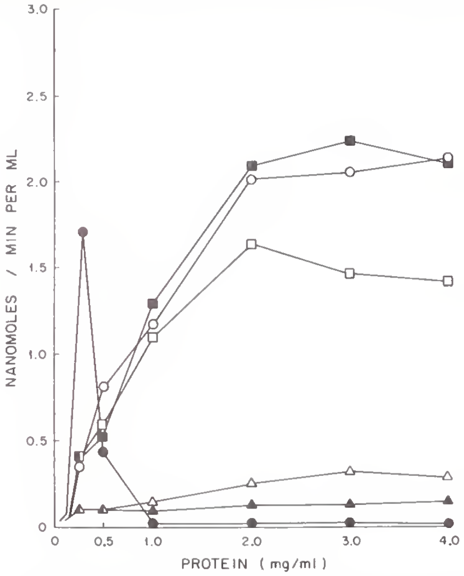

FIGURE 2.—BP metabolite formation as a function of microsomal protein. Metabolites were prepared and analyzed as in the "Materials and Methods" section and in the legend to figure 1; hepatic microsomes were from a single male *S. chrysops*. Of the total BP, 38 and 57% were metabolized in 10 min at protein concentrations of 1 and 4 mg/ml, respectively. Symbols: ●, 9-OH-BP; ○, BP-9,10-dihydrodiol; ■, 3-OH-BP; □, BP-7,8-dihydrodiol; ▲, BP-1,6-quinone; and △, BP-3,6-quinone.

TABLE 2.—*BP metabolites formed by scup microsomes from liver, kidney, and gill*[a]

Metabolite	Metabolites formed by:		
	Liver	Kidney	Gill
9,10-Dihydrodiol	341 (29)	71 (31)	9 (28)
4,5-Dihydrodiol	ND[b]	ND	ND
7,8-Dihydrodiol	331 (28)	49 (22)	8 (25)
1,6-Quinone	77 (6)	7 (3)	2 (6)
3,6-Quinone	108 (9)	6 (3)	1 (3)
9-OH-BP	32 (3)	15 (6)	0.5 (1)
3-OH-BP	307 (26)	80 (35)	11 (34)
Total activity, nmol BP transformed/min per mg protein	1.19	0.228	0.032

[a] Values are picomoles of product per minute per milligram protein with percent of total *in parentheses*. Data for liver were obtained with liver pooled from 2 fish. Values for kidney and gill represent data obtained with tissues pooled from more than 20 fish. Volume of reaction mixtures was 0.25 ml with protein concentrations of 0.5, 1.9, and 3.1 mg/ml for liver, kidney, and gill, respectively. The percent of total BP metabolized during incubations was 8.5, 2.0, and 0.5% for liver, kidney, and gill, respectively. Replicate assays varied by less than 15%.

[b] ND = not detected.

tissues may be important in the determination of target organs for such carcinogens. Previous studies with cardiac microsomes from scup disclosed that BP metabolism by either atrium or ventricle proceeded with a strong preference for the 7,8- and 9,10-positions (*19*). Examination of kidney and gill microsomes from scup revealed a similar metabolic capacity in these organs (table 2). Both formed a pattern of BP metabolites quite like that formed by scup liver microsomes, with between 50 and 60% of the total ethyl acetate-soluble metabolites being chromatographed with BP-9,10-dihydrodiol and BP-7,8-dihydrodiol. Microsomal preparations of these organs further resembled those from liver of the same species in their apparent inability to form BP-4,5-dihydrodiol or BP-6,12-quinone at the limits of detection. The overall rates of metabolism seen here with scup kidney and gill were like those we reported earlier (*16*).

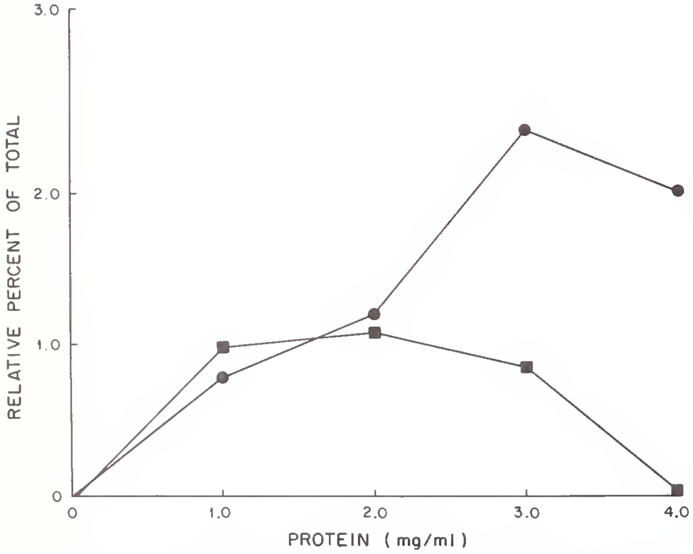

FIGURE 3.—Formation of metabolites eluting before 9,10-dihydrodiol as a function of microsomal protein. Metabolites were obtained as stated in legend to figure 2. Symbols: ■, retention time 2.9 min; ●, retention time 4.2 min Retention time of BP-9,10-dihydrodiol was 4.9 min.

Regiospecific patterns of BP metabolism with efficient formation of benzo-ring dihydrodiols, described above for several scup tissues, have been seen with hepatic preparations from other teleosts as well. More than 40% of the metabolites formed by mature male winter flounder (*P. americanus*) and killifish (*F. heteroclitus*) liver microsomes eluted with BP-9,10-dihydrodiol and BP-7,8-dihydrodiol (table 3). As with scup, little BP-4,5-dihydrodiol or 6,12-quinone were formed by either sample.

We assessed patterns of metabolism of BP by early developmental stages of fish using eleutheroembryos of *F. heteroclitus*. Microsomes prepared from whole embryos metabolized BP with a regiospecificity similar to that of adult liver microsomes of the same species (table 3). As with hepatic preparations, incubation of the embryo microsomes with BP and the epoxide hydrolase inhibitor trichloropropene oxide abolished the formation of the dihydrodiol derivatives.

DISCUSSION

The results here and those of previous studies (*12–14, 18, 20*) clearly indicate that microsomal preparations of liver from untreated teleosts commonly form high percentages of benzo-ring dihydrodiols of BP, a situation generally

TABLE 3.—*BP metabolites formed by microsomes from adult and embryonic fish tissues*

Metabolite	Percent of total formed by:		
	P. americanus[a]	*F. heteroclitus*[b]	
	Adult liver	Adult liver	Embryos
9,10-Dihydrodiol	21	19	21
4,5-Dihydrodiol	<1	ND[c]	ND
7,8-Dihydrodiol	21	20	24
1,6-Quinone	10	12	11
3,6-Quinone	7	15	23
9-OH-BP	6	5	7
3-OH-BP	35	29	14

[a] Liver microsomes from adult winter flounder were prepared from a single adult male; 100% activity was 0.147 nmol transformed/min per mg protein. Reaction mixtures of 1.2 ml contained 0.5 mg/ml. Less than 5% of total BP was metabolized in 15 min.

[b] Adult liver microsomes were prepared from a pool of 5 mature male fish. Embryo microsomes were from a pool of 172 eleutheroembryos (stages 35–37) treated with PCB. One hundred percent activity was 0.440 nmol transformed/min per mg protein for adults and 0.074 nmol/min per mg for embryos. Reaction mixtures were 1.0 ml containing 5 mg/ml and 0.1 ml containing 0.15 mg/ml for adult and embryo samples, respectively. Of total BP, 8.5% was metabolized in 15 min by adult samples, and less than 4% was metabolized in 30 min by embryo samples. Replicate assays varied by less than 15%.

[c] ND = not detected.

distinct from that in mammalian liver. The mass spectral analyses here unequivocally confirm the identity of these important products. The present results also establish that some extrahepatic tissues of fish form suites of BP metabolites like those seen with numerous teleost liver samples. Like comparisons based on hepatic metabolites, both gill and kidney microsomes from at least one fish species apparently form much greater percentages of benzo-ring dihydrodiols than do those of mammalian extrahepatic tissue, notably those from lung and kidney (*21*). The similarity of BP metabolite profiles in hepatic and extrahepatic tissues is relevant to the results with *F. heteroclitus* eleutheroembryos, which were prepared from whole individuals. If the features of BP metabolism identified with eleutheroembryos of *F. heteroclitus* are common to these stages of many teleosts, as is apparently true of adult liver, then the finding that eleutheroembryonic tissues form substantial amounts of BP-7,8-dihydrodiol is significant to the use of teleost embryos in carcinogenesis studies (*22*) and perhaps to the appearance of environmentally induced tumors in fish. However, the patterns of metabolism during earlier stages of development remain to be determined.

The patterns of BP metabolism described for fish clearly do not depend on the overall catalytic activity of a given sample. The overall activity in several of the samples here varied by two orders of magnitude, yet the metabolite profiles were remarkably conservative. The same phenomenon has been noted in other recent comparisons between fish species and between control and induced members of the same species (*14, 18, 20*). The latter point is significant to the present results because many of the fish here, although untreated, were not necessarily uninduced (*23*). Although the specificity for the benzo ring does not seem to be strongly associated either with higher activity or with induced cytochromes P-450 in fish when assessed with intact microsomes, this remains to be confirmed with purified forms of cytochrome P-450 from fish. It is known that those mammalian P-450 induced by aromatic hydrocarbon-type inducers are more efficient at forming benzo-ring metabolites of BP (*9, 10*) and at further metabolizing BP-7,8-dihydrodiol to bay-region diol epoxides, whether or not these cytochromes P-450 catalyze a greater turnover of BP (*24*). These same induced cytochromes P-450 are apparently efficient at metabolizing, with a high degree of stereoselectivity, other PAH that have bay regions to dihydrodiols having an adjacent double bond in the bay region (*25–27*). We might predict that the regiospecific pattern of metabolism of BP by many teleosts, which is like that of induced mammalian cytochrome P-450, will be reflected in profiles with other PAH as well and that these patterns will be accompanied by stereoselectivity. The appearance of such structural restrictions in the metabolism of many hydrocarbons by fish would have implications 1) for the nature and origin of some cytochromes P-450 in marine species, and 2) for the distinguishing of endogenously produced metabolites from many of those that might be exogenously produced and accumulated in environmental samples.

Numerous studies have clearly indicated that a principal pathway to an active carcinogenic form of BP involves the metabolism of $(-)$-BP-7,8-dihydrodiol to a particular bay region diol epoxide enantiomer that binds to DNA (*3, 25, 28*). Ahokas and associates (*12*) and Varanasi and Gmur (*14*) demonstrated the in vitro binding of BP to DNA catalyzed by teleost hepatic preparations. Moreover, Ahokas et al. (*12*) determined that formation of one of the major DNA adducts was catalyzed by trout (*Salmo trutta lacustris*) microsomes and was that of a BP-7,8-diol-9,10-epoxide. The significance of such metabolism and adduct formation to carcinogenesis studies with fish remains to be established by in vivo studies. However, Varanasi and Gmur (*29*) obtained evidence that BP-7,8-dihydrodiol was a prominent oxygenated derivative formed in vivo that could be found in bile and tissues of several species of marine fish and that BP can be covalently bound to fish liver DNA in vivo (*30*). Defining the extent to which BP is metabolized to known carcinogens and the extent to which they are active in vivo will require precise identification of the enantiomeric forms of diol epoxides and the specific adducts with DNA formed in vivo in fish.

Clearly, tumorigenesis is not a required concomitant of patterns of BP metabolism like those identified for fish in vitro. In fact, few (*31, 32*) have been successful in attempts to induce tumors in fish with known carcinogenic PAH. Inasmuch as the regiospecific patterns of BP metabolism by varied fish organs are consistent with patterns known to result in the formation of mutagens and carcinogens, some other factors may be responsible for the limited success in experimental induction of neoplastic disease with BP or other PAH in fish. Factors related to 1) the site of production of metabolites in the cell, 2) the half-life of reactive intermediates in vivo, 3) the persistence of specific DNA adducts, and 4) the survival of any transformed cells could all be involved in the determination of the expression of carcinogenic potential. Thus the role of enzymes of nuclear as opposed to microsomal membranes (*33*) in metabolizing PAH in fish, the significance of alkylation of mitochondrial DNA (*34*), the role of conjugating enzymes in scavenging reactive metabolites in vivo, the capacity for DNA repair, and the function of immune systems could all be factors in the carcinogenicity of PAH in fish. It would seem that fish might be used fruitfully in comparative studies of the role some such factors play in oncogenesis. Such research should also define the significance of PAH to environmental cancers in fish.

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DISCUSSION

G. Bailey: Dr. Stegeman, we have information from other systems that demonstrates that, when one is dealing with in vitro extracts, the pattern of BP metabolism one sees can be affected or resolved by the concentration of BP that is put in the system, and that, of course, makes it difficult to extrapolate high concentration in vitro studies back to what may be happening in vivo during the carcinogenic process. Can you comment on that? Have you examined the influence of pyrene concentration, or was I thinking something else when you gave your data? Have you studied that effect?

J. J. Stegeman: We have not looked at the substrate-dependent profile. We are doing that right now with these animals. The only instances in which we studied substrate dependence of metabolite production is in the production of mutagenic derivatives, i.e., the introduction of reaction metabolites in the in vitro system, and that profile is linear up to the point at which the curve breaks.

J. Schultz: Once an enzyme is induced, how long does it take for its level to decline again?

Stegeman: It varies from species to species, I would think. Dr. Bend has done more of that than we have, and I would like him to comment on that.

J. Bend: It is highly dependent on temperature. At temperatures in Maine, you will be talking about at least 3 days and often up to 5 or 6 before you will see optimal induction. At that cold temperature, the longest we have kept animals is 90 days and they are still induced. In Florida, the water temperature is around 24° C or approximately that on the Gulf Coast.

Stegeman: Yes, I think it gets up as high as 26° C. You are talking about 24 to 48 hours to maximum induction and down after 15 days; it is highly dependent on water temperature. I am referring to single doses of inducer.

Bend: Yes, the onset of induction is also dependent upon the temperature.

J. Couch: The rat fish you showed was most interesting, but I think it is not too surprising that the rat fish, which is treated at the bottom of the sink, might have high induction. By chance, was the fish taken from anywhere near the New York bight?

Stegeman: Well, the New York bight, strictly speaking, does not extend out that far. The fish were taken close to the Hudson Canyon, however.

Coach: That fish is no more protected in the deep water?

Stegeman: Oh, absolutely not. We first found this with deep sea fish in 1978. The fish that I showed you the data for were collected in the spring of this year (1981).

Couch: Maybe if we looked at more truly deep water specimens, we would really find out what is going on in the ultimate recipient.

Stegeman: We have in mind a study to try to map geographically the areas where there is evidence for high induction and perhaps to find out what the causes are. You know, if natural products are the cause, then we should be able to find areas with these natural products. Chemists can then tell us that induction is likely or not likely, or induction by this or that compound is likely or not likely. In addition to substances like the flavonoids that Dr. Bend mentioned, there are aromatic hydrocarbons that are produced in situ by diagenetic processes in recent sedimentary deposits. Perylene is one that is produced by transformation of material that comes from diatoms, and this appears as a big peak in many areas and in large concentrations.

Couch: In a pollution sense, that does not necessarily make a remote environment because of the amount of ocean dumping that is prevalent.

Stegeman: No, it is just remote for sample retrieval.

Couch: The other point was related to your embryo studies. You were using *Fundulus,* basically, in those embryo studies, and you did histology on embryos to determine origin of liver, time of origin of liver bud, and liver development.

Stegeman: Well, we were right on the cusp, I think, and so I said there was no liver present. The veterinary pathologist looked at these samples for us.

Couch: Unless you serially section the embryo all the way through, you could possibly miss the liver.

Stegeman: He did serially section it.

Couch: There is a good chance that some liver might be in those animals at that time.

Stegeman: As I say, we were right on the cusp. I hesitate to say that there was no liver, but that is what histological analysis indicated.

Dietary Modification of Aflatoxin B_1 Carcinogenesis: Mechanism Studies With Isolated Hepatocytes From Rainbow Trout[1, 2]

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Janet S. Wilcox,[3] Russell O. Sinnhuber,[3] *and* Daniel P. Selivonchick[3]

ABSTRACT—Hepatocytes were prepared from rainbow trout by perfusion in situ with collagenase and hyaluronidase. Preparations normally showed high initial viability (95 ± 5% dye exclusion, 92 ± 5% lactate dehydrogenase retention) and gradually decreased in viability and glutathione concentration over 5 hours. Cellular metabolism of aflatoxin B_1 (AFB_1), a potent hepatocarcinogen, was characterized by an investigation of the following parameters: kinetics of AFB_1 metabolism and DNA adduct formation, dose response, viabilities of detoxication and activation pathways with time, influence of organic solvents, and effect of variation in cell concentration. The AFB_1 metabolites and DNA adducts were resolved and quantitated by high-performance liquid chromatography. From these results a standardized assay procedure was derived which we used to examine AFB_1 metabolism and DNA adduct formation in hepatocytes from fish fed dietary substances known to alter the carcinogenic response to this mycotoxin. Dietary β-naphthoflavone, which strongly inhibits AFB_1 carcinogenesis in rainbow trout, dramatically and reproducibly altered AFB_1 binding and metabolism in isolated hepatocytes. Overall rate of AFB_1 metabolism and rates of detoxication reactions increased, whereas DNA binding decreased. Dietary cyclopropenoid fatty acids, powerful synergists and promoters of AFB_1 carcinogenesis in trout, also repressed AFB_1–DNA binding. Both dietary factors appeared to depress initial DNA damage by AFB_1 but operated through different metabolic pathways to do so. — Natl Cancer Inst Monogr 65: 379–385, 1984.

Marine and freshwater fish species are receiving increasing attention for possible in situ screening of water-borne environmental pollutants and as alternate vertebrate models for the screening of potential carcinogens. The acceptability and impact of any fish model for carcinogenesis studies will be defined and limited by the amount of comparative information which relates its biologic response to that in mammals. Comprehensive comparative studies would examine: 1) comparative pharmacokinetic distribution of xenobiotics, including variation in dose and route of administration; 2) target organ specificity and sensitivity to known mammalian carcinogens; 3) target organ metabolism, activation, and detoxication characteristics, including types of mixed function oxidase activities and their response to known mammalian inducers of the enzyme; 4) characteristics of genome damage and repair; 5) comparative histology and pathology of lesions; and 6) influence of genetic variability on population and individual response. Therefore, selection of suitable fish models will involve balanced choices according to sensitivity and appropriateness of the model to the problem being investigated, extent of comparative information available or obtainable, and total per animal cost of the screening test (including husbandry requirements).

The rainbow trout has been extensively developed as a fish model for carcinogenesis studies. Trout have been shown to be sensitive to a range of carcinogens including mycotoxins, benzo[*a*]pyrene, *N*-dimethylnitrosamine, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [(*1–3*); Sinnhuber RO, Bailey GS, Nixon JE, et al: Unpublished results]. Tumor histologies and pathologies have been described (*4*), as have the characteristics of liver mixed function oxidases and their response to inducers (*5, 6*). A measurable dose of carcinogen can be conveniently administered to rainbow trout by brief exposure of fertilized embryos (*7*), by the feeding of juveniles, or ip injection. An extensive literature also exists that describes nuclear protein sequences (*8, 9*), chromatin structure, and developmental transitions (*10, 11*), all of which provides a background for studies in carcinogenesis at the detailed molecular level (*12*) in this species.

In addition to whole animal studies, the metabolism, detoxication, and initiation of genome damage of suspect carcinogens can be examined in isolated target organ cells. Techniques for isolation of hepatocytes from rainbow trout have recently been described (*13–15*), and hepatocyte metabolism of AFB_1, a model hepatocarcinogen, has been characterized (*15*). The results of our studies on the influence of selected dietary factors on AFB_1 metabolism and genome damage in isolated trout hepatocytes show

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ABBREVIATIONS: AFB_1 = aflatoxin B_1; HPLC = high-performance liquid chromatography; AFM_1 = aflatoxin M_1; β-NF = β-naphthoflavone; CPFA = cyclopropenoid fatty acid(s).

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that factors which alter AFB_1 tumorigenesis in vivo can substantially alter AFB_1 metabolism and initial genome damage in isolated hepatocytes. Thus isolated hepatocytes offer promise as a useful vertebrate cell system for in vivo screening of suspected hepatocarcinogens, for characterization of their metabolism and macromolecular interactions, and for examination of the mechanisms by which extrinsic factors (e.g., dietary predisposition, water quality, genetic variation, temperature, etc.) might influence the carcinogenic process at the cellular level.

MATERIALS AND METHODS

Chemicals.—We examined AFB_1 (Calbiochem, San Diego, Calif.) for purity by UV spectrometry and thin-layer chromatography on silica gel (benzene–acetone–ethyl acetate, 55:15:30). We analyzed $[^3H]AFB_1$ (Moravek Biochemicals, Inc., Brea, Calif.) by radioscanning after thin-layer chromatography on silica gel (chloroform–acetone, 9:1). Rainbow trout were reared from brood stock at the Oregon State University Fish Toxicology and Nutrition Laboratory and are an outcrossed Shasta strain derived over 18 years for carcinogenesis studies (*1–4*).

Hepatocyte preparation.—The procedure, described in detail elsewhere (*15*), is a modification of that described by Hazel and Prosser (*13*). A two-step in situ perfusion with collagenase and hyaluronidase was done at 23° C; the procedure was standardized to provide hepatocytes 105± 10 minutes after the trout have been killed. Viability of hepatocytes was routinely determined by lactate dehydrogenase leakage (*16*), glutathione content (*17*), and trypan blue exclusion. Cell concentrations were estimated by hemocytometry, and DNA analysis was by the Burton method (*18*).

Hepatocyte incubations with AFB_1.—We used the same buffers and procedures for hepatocyte incubation as in our previous studies (*15*), in which 5-ml final reaction volumes contained $2\text{–}6 \times 10^6$ cells/ml and incubations were started by the addition of a freshly prepared aqueous solution of $[^3H]AFB_1$ (4 μCi, 3.4 μg), freed of ethanol solvent by prior evacuation treatment (*15*). Reactions were conducted for 60 minutes at 23° C with both continuous shaking and gassing with 95% O_2:5% CO_2. Cells were rapidly centrifuged, washed, frozen with liquid nitrogen, and stored at −80° C. Each sample supernatant and wash was combined, acidified with 20 μl acetic acid, and stored at −80° C.

Procedures for DNA isolation and specific activity and AFB_1 metabolite determinations by HPLC are described in (*15*).

RESULTS

Hepatocyte Preparation

In the electron micrograph of freshly isolated hepatocytes shown in figure 1, the cells are rounded after collagenase release and show good integrity of organelles, such as mitochondria and endoplasmic reticulum. Initial cell viability is routinely high in these preparations (90–95% by lactate dehydrogenase retention) and declines slowly with the cellular glutathione (fig. 2) as the preparation ages.

Metabolism of Aflatoxin B_1 in Isolated Hepatocytes

The major unbound metabolites resulting from AFB_1 incubation in isolated hepatocytes were resolved and quantitated by HPLC (fig. 3). The metabolites of AFB_1 in these cells include a series of polar conjugates of AFB_1, not yet individually characterized (fig. 3, region 1), aflatoxicol M_1 (peak 2), AFM_1 (peak 3), unreacted AFB_1 (peak 5), and aflatoxicol (peak 6). The ratios of these peaks are shown below to vary according to dietary pretreatment of the fish. (The peaks in region 4 are not characterized but appear to be artifacts of AFB_1 degradation which do not vary in a systematic way with diet.)

A number of parameters which might influence the relative apportioning of AFB_1 metabolites and the formation of DNA adducts have been reported (*15*) and are summarized in table 1. In these isolated cells, incubation with AFB_1 produces DNA adducts similar to those formed in vivo (*19*), in amounts linearly related to dose. Overall AFB_1 reaction rate is also linear with dose, and the relative apportioning to aflatoxicol, AFM_1,[5] and conjugates is also dose independent. In contrast, relative patterns of metabolite formation and rate of adduct formation[6] are significantly altered when incubations contain over 10^7 cells/ml, in preparations beyond 1 hour of age or in the presence of the common solvent vehicles ethanol or dimethyl sulfoxide.

These results have provided the basis for our standard hepatocyte assay conditions, which utilize fresh cells between 0 and 1 hour old, a cell concentration of $2\text{–}6 \times 10^6$ cells/ml, and reaction times of 15 minutes to 1 hour at 23° C. Solvents for carcinogen are removed from solution before cells are added. Reactions can be corrected by doses in the known linear range of 0.06–6 μg AFB_1/ml.

Influence of Dietary β-Naphthoflavone on Aflatoxin B_1 Metabolism and DNA Adduct Formation in Isolated Hepatocytes

Dietary β-NF is known to reduce AFB_1 hepatocarcinogenic response when fed before and with AFB_1 exposure (*20*). One possible mechanism for inhibition might be through alterations in AFB_1 metabolism in liver cells in vivo which lead to decreased initial DNA damage by the carcinogen. To test this, we isolated hepatocytes from fish fed β-NF or the control diet and examined them by pairs for rates of AFB_1 metabolite and DNA adduct formation. The results of 7 such incubation experiments are shown in table 2. The most dramatic effect was the tenfold to twentyfold elevation in conversion of AFB_1 to the less carcinogenic AFM_1. Conversely, formation of the equally carcinogenic (*21, 22*) aflatoxicol was reduced threefold.

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[5] Most of the data presented here on AFB_1 metabolites were derived from an HPLC program (*15*), which did not resolve aflatoxicol M_1 and AFM_1; hence these are reported together as AFM_1. Aflatoxicol M_1 is present in significant amounts only in hepatocytes from β-NF-induced fish, at approximately 30% of the total aflatoxicol M_1 plus AFM_1 peaks, and thus represents another major change in AFB_1 metabolism due to dietary β-NF.

[6] We have not determined the rate of ongoing adduct repair in isolated trout hepatocytes. The data we present here as adduct formation rates may more properly be termed accumulation rates if repair should prove significant.

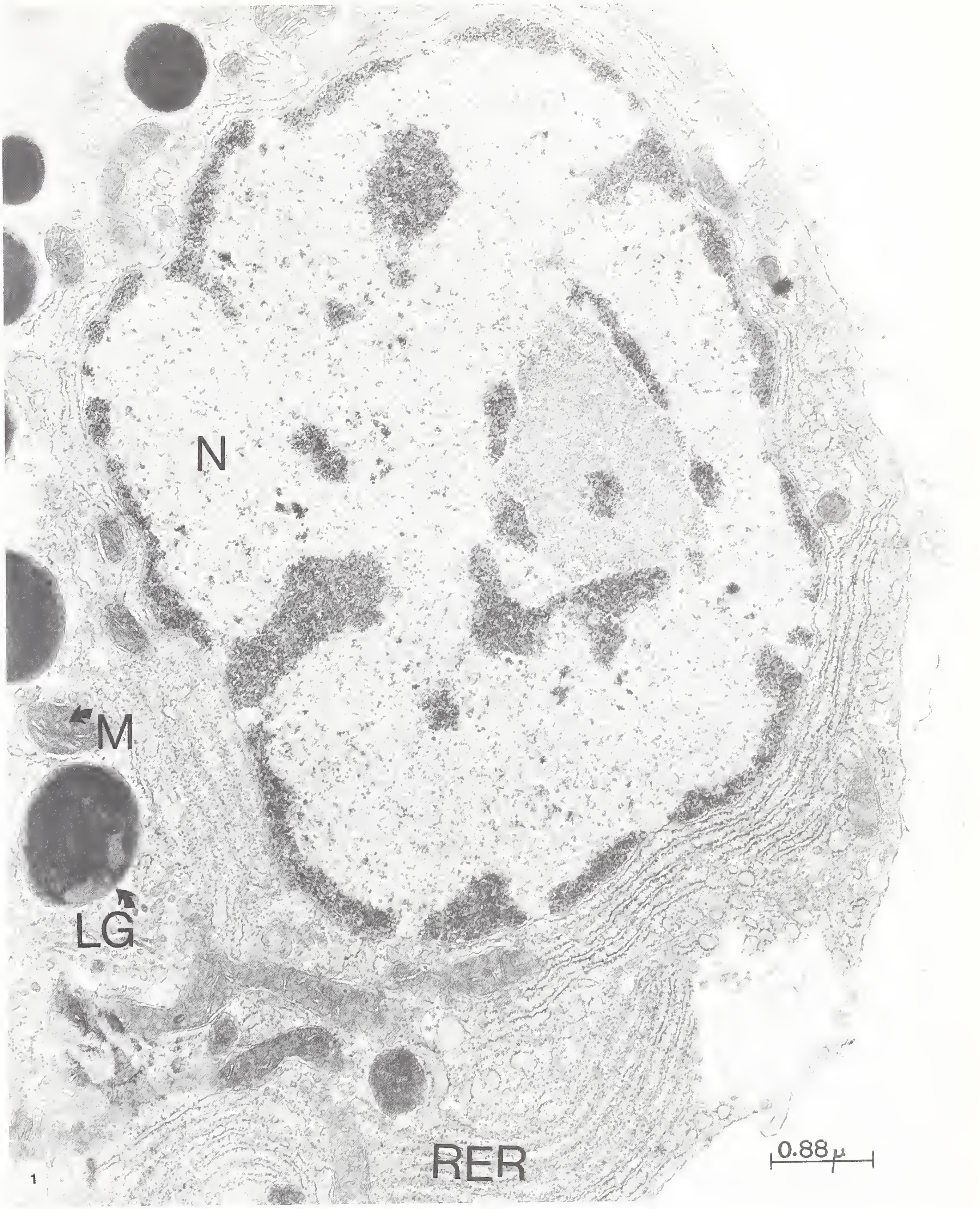

FIGURE 1.—Electron micrograph of freshly isolated rainbow trout hepatocytes from fish reared on a control diet. Hepatocytes were fixed in ice-cold 2.5% glutaraldehyde in phosphate buffer at pH 7.2 for 1 hr, rinsed in buffer, postfixed in 1% osmium tetroxide in phosphate buffer for 1 hr and dehydrated in a graded series of ethanol. Material was then stained with 1% uranyl acetate before infiltration and embedding in Spurr's low viscosity medium and examined with a Philips electron microscope. *Note* dense arrays of rough endoplasmic reticulum (RER), well-defined nucleus (N), mitochondria (M), and presence of lipid globules (LG).

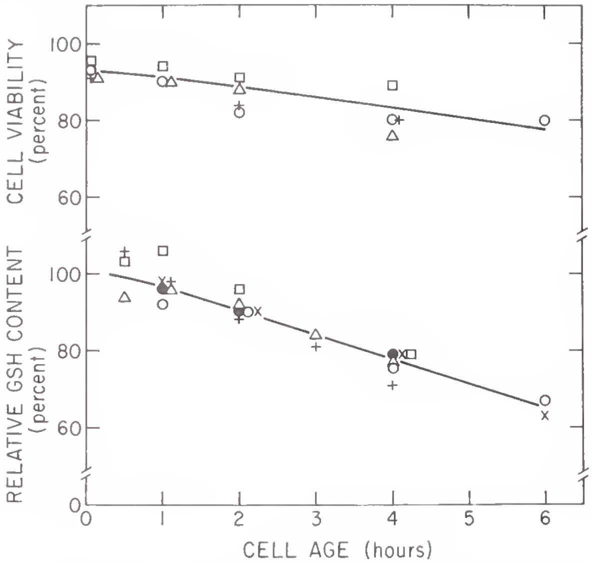

Figure 2.—Viability and glutathione content of isolated hepatocytes. Viability was determined by lactate dehydrogenase leakages; *symbols* represent determinations for 4 preparations, except glutathione determinations represent 6 preparations. Amino acid supplements were not added to the medium in these particular preparations. GSH = glutathione.

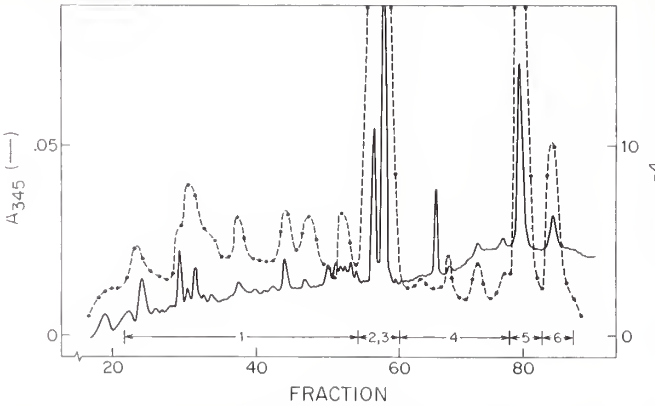

Figure 3.—An HPLC profile of [^{3}H]AFB$_1$ metabolites in hepatocytes from a trout fed a diet containing β-NF. Samples were prepared from aqueous hepatocyte incubation medium as described (*20*). Resolution was obtained on a μBondapak C$_{18}$ column (3.9 × 300 mm, Waters Associates, Milford, Mass.) by linear 30-min gradient elution with 10 to 30% acetonitrile in 0.02 *M* potassium acetate, pH 5.0. Flow rate was 1.0 ml/min, chart speed was 1 cm/min. Thirty-drop fractions were collected for liquid scintillation counting; UV detection was at 345 nm, with sensitivity at 0.01 absorbancy full scale. DPM = disintegrations/minute.

Formation of polar conjugates was either unaffected or mildly stimulated. The DNA adduct formation was reduced to about 60% of the average value in control hepatocytes. The overall rate of AFB$_1$ metabolism, as measured by the amount of it recovered in unreacted form, was elevated in β-NF hepatocytes.

The influence of β-NF on AFB$_1$ reaction rate and AFM$_1$ and aflatoxicol formation are all statistically significant ($P \leq 0.05$) for each of the 7 paired experiments. Conjugate formation was enhanced in 5 of 7 experiments but only 3 of these are significant. Again, DNA adduct formation was reduced in all 7 paired experiments though 2 of these are not significant. If the cumulative *t*-test for paired differences is applied to the 7 experiments, then all the effects of β-NF are highly significant. These results show that dietary β-NF leads to reproducibly altered metabolism of AFB$_1$ in isolated hepatocytes in which detoxication reactions are favored and DNA adduct accumulation is reduced.

Influence of Dietary Cyclopropenoid Fatty Acids on Aflatoxin B$_1$ Metabolism and DNA Adduct Formation in Isolated Hepatocytes

Dietary CPFA are synergistic for AFB$_1$ carcinogenesis (*20*) and depress rather than elevate mixed function oxidase activities. Hepatocyte studies similar to those for β-NF were done to see whether these dietary fatty acids might be synergistic by virtue of alterations in AFB$_1$ metabolism that lead to enhanced DNA damage by AFB$_1$. The results in table 3 show that dietary pretreatment with CPFA

Table 1.—*Summary of parameters which influence AFB$_1$ metabolism and DNA adduct formation in isolated hepatocyte incubations*

Parameter	Relative distribution of major unbound AFB$_1$ metabolites[a]	AFB$_1$ reaction, rate/cell	DNA adduct accumulation, rate/cell
AFB$_1$ dose	Percentages unchanged	Proportional to dose	Linear with dose, 0.06–6 μg/ml
Preparation age			
First hr	Percentages unchanged	Constant	Constant
After first hr	Percentages altered	Increases, then decreases	Increases, then decreases
Hepatocyte concentration 0–6 × 10^6/ml	Percentages unchanged	Decreases exponentially	Decreases slightly
>10^7/ml	Percentages altered	Decreases exponentially	Decreases sharply
Solvent vehicles present (0.25–4%)	All equally inhibited by ethanol, aflatoxicol not inhibited by dimethyl sulfoxide	Decreases	Decreases sharply

[a] Metabolites include conjugates, AFM$_1$, and aflatoxicol.

TABLE 2.—*AFB_1-DNA binding and metabolite distribution in hepatocytes from trout fed control and β-NF diets*[a]

Paired experiment	Distribution of recovered unbound isotope				DNA sp act[b]
	Conjugates	AFM_1	Aflatoxicol	AFB_1	
1 Control	18±0.9[c]	1.5±0.2	16 ±0.6	53±0.7	1,295±218
β-NF	18±0.9	36 ±0.6	5.0±0.4	31±1	539± 16
Significance[d]	NS	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.025$
2 Control	24±5	1.8±0.2	14 ±1	46±3	1,319±112
β-NF	24±3	31 ±1	4.8±0.8	29±0.6	470± 17
Significance	NS	$P<0.001$	$P<0.005$	$P<0.01$	$P<0.005$
3 Control	16±4	1.6±0.5	14 ±2	56±3	1,679±116
β-NF	25±4	21 ±2	5.3±0.1	36±0.8	1,469±161
Significance	NS	$P<0.005$	$P<0.025$	$P<0.01$	NS
4 Control	13±0.9	1.6±0.3	17 ±0.5	57±1	1,163±60
β-NF	16±1	15 ±0.2	7.1±0.5	50±2	626±13
Significance	$P<0.05$	$P<0.001$	$P<0.005$	$P<0.025$	$P<0.005$
5 Control	22±2	2.1±0.3	19 ±0.9	45±4	1,235±171
β-NF	32±2	30 ±3	5.0±2	23±7	697± 42
Significance	$P<0.01$	$P<0.001$	$P<0.01$	$P<0.05$	$P<0.025$
6 Control	22±1	2.3±0.4	17 ±2	36±4	1,429±186
β-NF	28±1	20 ±0.8	7.0±2	16±2	1,236± 59
Significance	$P<0.025$	$P<0.001$	$P<0.025$	$P<0.025$	NS
7 Control	26±2	1.4±0.2	15 ±1	23±3	1,401±227
β-NF	31±1	36 ±2	2.4±0.1	14±1	916± 33
Significance	NS	$P<0.001$	$P<0.005$	$P<0.025$	$P<0.05$
Cumulative significance (paired difference, *t*-test, $n=7$)	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$

[a] NS = not significant.

[b] Specific activity is expressed in disintegrations per minute $[^3H]AFB_1$ per microgram DNA.

[c] Values are means ± SD for triplicate samples. Metabolites are expressed as a percentage of total disintegrations/minute applied to HPLC columns.

[d] Significance is by unpaired *t*-test, $n=3$.

results in hepatocytes with altered AFB_1 metabolism compared with controls. Formation of conjugates and of AFM_1 were depressed (table 3), whereas the overall AFB_1 reaction rate was unaffected (data not shown). By contrast, aflatoxicol formation was stimulated, and comprised the major unbound metabolite of AFB_1 in CPFA hepatocytes. These metabolic changes are accompanied by a reduction in DNA adduct formation to an even greater extent than that shown by β-NF pretreated fish. These studies in isolated cells clearly do not support the hypothesis that dietary CPFA are synergistic by enhancing genome damage by AFB_1.

TABLE 3.—*Effects of dietary CPFA on AFB_1 metabolism and DNA adduct formation in isolated hepatocytes*

Diet	Distribution of unbound AFB_1 metabolites			DNA sp act[a]
	Conjugates	AFM_1	Aflatoxicol	
Control ($n=5$)	36±3.8[b]	6.9±2.1	57±4.5	2,090±770
CPFA[c] ($n=9$)	21±5.6	3.0±0.8	76±5.5	580±320
Significance[d]	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$

[a] *See* explanation in footnote *b*, table 2.

[b] Numbers are means ± SD of 9 CPFA and 5 control fish preparations, in which each preparation received 3 to 6 replicate incubations with $[^3H]AFB_1$ (7.5 μCi, 3.8 μg) in the standardized assay.

[c] Fish were fed 300 ppm dietary CPFA as *Sterculia foetida* oil for 6–8 weeks before hepatocyte preparation.

[d] Significance was determined by *t*-test for unpaired differences; degrees of freedom = 12, and $t=4.94$, 4.59, 6.1, and 4.72, respectively.

DISCUSSION

Procarcinogen Metabolism in Isolated Cells

Increasing evidence shows that studies of procarcinogen metabolism and DNA adduct formation in intact isolated cells from rodents more accurately reflect in vivo metabolism than do studies in which broken cell preparations are used (*23–25*). This accurate reflection is especially true for compounds subject to a number of competing oxidation, reduction, and conjugation reactions, in which the loss of either compartmentalization, important cofactors, enzyme viability, or all three may seriously distort the total metabolism pattern. The results presented here and elsewhere (*15*) demonstrate that hepatocytes isolated from rainbow trout show similar promise for the examination of xenobiotic metabolism in vitro. These cell preparations exhibit good initial viability, maintenance of various AFB_1 metabolic pathways adequate for 1-hour assays, and formation of DNA adducts similar to those formed in vivo.

Especially important is the demonstration that dietary-induced alterations in AFB_1 metabolism and binding are reproducible between individual fish preparations. For each of 7 experiments described here, hepatocytes from a β-NF pretreated fish showed markedly enhanced AFM_1 formation and significantly reduced aflatoxicol formation compared with control hepatocytes; DNA adduct formation was also always reduced (though the differences from control were not significant in 2 experiments). We interpret these results to suggest that isolated hepatocytes retain, at least partially, their in vivo characteristics of AFB_1 metabolism and adduct formation.

Although cultured cell lines may provide a more convenient source for in vitro metabolism studies, such lines often show altered characteristics in metabolism, response to inducing agents, and enzyme levels (*26-28*). When it is important for one to relate in vitro results to cellular metabolism in vivo in the parent cell type, freshly isolated hepatocytes may be preferable.

Hepatocyte Use in Studies on Carcinogenesis Mechanisms

Although the above results indicate the usefulness of isolated hepatocytes for the study of metabolism at the cellular level, one must exercise caution in extrapolating such results to predict carcinogenic response in the whole animal. The in vivo effects of any factor such as dietary β-NF on carcinogenic response will be the net result of its effects on procarcinogen delivery and uptake by target organs, metabolism and binding within the target cells, rate of organ and systemic elimination of partially detoxified metabolites, rate of repair of potentially transforming lesions, and probability of transformation per surviving lesion. In the results we presented here, only cellular metabolism and binding, as influenced by dietary compounds known to modulate AFB_1 hepatocarcinogenesis, were examined.

For dietary β-NF, these hepatocyte studies suggest that inhibition of AFB_1 carcinogenesis may occur partly as a result of alterations in AFB_1 metabolism at the cellular level that lead to reduced initial genome damage. This is supported by the observation that dietary β-NF strongly induces mixed function oxidase activities in trout liver (*20*). However, the studies outlined above must also be performed before the mechanisms of β-NF inhibition are fully known. The importance of this is emphasized by the results with dietary CPFA. Although these lipids are strongly synergistic for AFB_1 hepatocarcinogenesis (*20*), they do not induce elevated AFB_1–DNA binding in isolated hepatocytes but reduce it instead. This apparent contradiction is resolved by studies which show that carcinogenesis initiated at the embryo stage in trout by AFB_1 is promoted by subsequent exposure to CPFA (*8*), possibly through alterations in membrane structure (*29*). Therefore, CPFA appear to enhance AFB_1 carcinogenesis during combined exposure experiments by reducing initial genome damage by AFB_1 but strongly promoting the probability of transformation from those reduced numbers of lesions. Such postinitiation promotional effects are unlikely to be detected or predicted by studies in hepatocytes alone.

In summary, isolated hepatocytes from rainbow trout are a promising system for the examination of metabolism of suspect procarcinogens, for comparisons of a range of compounds for their potential to damage DNA in a vertebrate cell system, and for the study of the short-term effects of added substances, such as antioxidants or vitamins. However, the relationship of such results to whole animal carcinogenic response will be apparent only if accompanied by further extensive studies on pharmacokinetics, repair, and postinitiation effects. A major attribute of the rainbow trout as an aquatic model for environmental carcinogenesis is that the entire range of such studies is experimentally accessible. This allows a more complete and direct comparison with mammalian carcinogenesis than is possible with small aquarium species. Against these advantages, one must recognize that the restrictive habitat and specialized laboratory requirements of rainbow trout may limit usefulness for in situ field monitoring programs and increase the cost, relative to aquarium species, for simple laboratory screening studies.

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DISCUSSION

J. Bend: I was intrigued by the excellent studies you have done, and the questions I have are only complimentary rather than critical.

Have you investigated the alkylation profiles in whole liver and compared the result with those created in hepatocytes?

G. Bailey: That is a good question. We have. In my abstract, I quoted some results about which I am now neither happy nor satisfied. We have taken fish which were on β-naphthoflavone and CPFA diets and given them ip injections of radiolabeled aflatoxin, waited a period (24 hr), which may be too long, and looked at adduct formation to see if there was any difference. The first time we did this experiment, β-naphthoflavone showed a reduction, and, in 1 experiment, CPFA showed no reduction and in another, some reduction.

We repeated that experiment just before this Conference, and I have to confess that I messed it up, but I would like to withhold any comments on that measurement of the pharmacokinetics until we repeat the experiment.

Bend: When you check the ratios of the differing adducts in the hepatocytes, do you always get the same ratios?

Bailey: If you do 1-hour reactions, you will get one pattern, whether you do that reaction between 0 and 1 hour of preparation age or 4- and 5-hour preparation age; however, if you do a 0- to 5-hour incubation, then you will see an increase in those ring open forms and a decrease in the N-7 adduct, which one would expect.

Bend: One final question. What is happening to your intercellular glutathione with time in the hepatocytes?

Bailey: That was on one of the slides that I showed. It drops only slightly, and we can deplete it, if we want, using diethyl maleate or other such substances. We have not examined the influence of that on glutathione conjugate formation, and we think direct identification of the conjugates themselves is a better route because you have to add diethyl maleate in some solvent; then you are in trouble right away.

Comparative Benzo[*a*]pyrene Metabolite Patterns in Fish and Rodents[1, 2]

Paul Melius[3, 4]

ABSTRACT—Benzo[*a*]pyrene is converted to 3-hydroxybenzo[*a*]pyrene, 9-hydroxybenzo[*a*]pyrene, benzo[*a*]pyrene-4,5-dihydrodiol, benzo[*a*]pyrene-7,8-dihydrodiol, benzo[*a*]pyrene-9,10-dihydrodiol, and benzo[*a*]pyrene quinones by postmitochondrial supernatant or microsomes in such fish as the rainbow trout, flounder, salmon, mullet, little skate, *Fundulus grandis,* and sea catfish. It is also now well-established that many fish convert benzo[*a*]pyrene to potent mutagenic metabolites as has been demonstrated with the Ames test, especially when the fish are induced with Aroclor 1254 or 3-methylcholanthrene. The metabolite patterns obtained at different substrate concentration levels indicate that the metabolism is more complex at low concentrations when metabolites are recycled in the in vitro system. — Natl Cancer Inst Monogr 65: 387–390, 1984.

The presence of MFO activity is now well-established in many different fish species (*1–5*). This type of enzymatic activity was initially demonstrated by measurement of aryl hydrocarbon hydroxylase activity, which usually involved measuring the fluorescence of certain of the metabolites such as 3-OH-BP during the metabolism of BP. With the advent of high-pressure liquid chromatography, techniques for separation and quantitative analysis of the metabolites of such compounds as BP (*6*) allowed the metabolite patterns of this compound to be determined in fish as had been done in the rat. A number of similarities occur in the types of oxygenated BP compounds produced by microsome preparations of fish and rats. The capability of a microsomal system to activate xenobiotics can also be assessed through biological end points. Thus through the use of the Ames test (*7*) for detection of mutagenic metabolites of BP, investigators can determine that fish microsomes, like those of the rat, metabolize BP to mutagens. In the following discussion, my use of the Ames test to detect BP mutagen formation by fish postmitochondrial preparations will be reviewed, and a comparison of the BP metabolite patterns generated by various fish and rat microsomal systems will be presented.

The sequence of BP metabolism in the rat initially involves formation of 2,3-, 4,5-, 7,8-, and 9,10-epoxide (*8*). The analysis of ethyl acetate extracts of microsomes or S-9 fractions incubated with BP indicates the presence of the 4,5-, 7,8-, and 9,10-dihydrodiols, which are supposedly due to the action of epoxide hydratase on the corresponding epoxides. The major phenol formed is the 3-OH-BP which is formed by an "NIH" shift mechanism of the 2,3-epoxide intermediate. The *trans*-7,8-dihydrodiol of BP formed in the rat liver is converted to the *trans*-7,8-dihydrodiol-9,10-epoxide, which reacts with DNA (*8*) and is believed to be the ultimate carcinogenic metabolite for BP. Thus, depending on exposure or physiological conditions, or both, within the rat, the following sequence can occur within the liver:

$$\text{BP} \xrightarrow{\text{MFO}} \text{7,8-epoxide} \xrightarrow{\text{epoxide hydratase}} \textit{trans}\text{-7,8-dihydrodiol} \xrightarrow{\text{MFO}}$$
$$\text{dihydrodiol-epoxide I and II} \rightarrow \text{DNA binding (mutation, cancer).}$$

Of course, phenols, other diols, and even triols and tetrols are formed from BP as well as the water-soluble conjugates.

The activity of the MFO system in the rat has been increased (induced) by polycyclic hydrocarbons and polychlorinated biphenyls (*9*). Liver microsomes from MCA-treated rats were ten times more effective in the production of diol epoxide I, compared with diol epoxide II from the *trans*-7,8-dihydrodiol. Thus the inducing agent has a profound effect on the type of MFO activity present in the microsomal fraction of the animal.

In studies on mullet (*Mugil cephalus*), Tan et al. (*10*) observed striking similarities with the rat in the in vitro metabolism of BP. The system described by Selkirk and associates (*6*) was followed for the in vitro studies on the conversion of BP in which hepatic S-9 fractions from Aroclor 1254-induced rats and mullet were used. The incubations were shaken for 30 minutes at 37° C under red light illumination. As in the rat, we found a marked increase (eightfold) in S-9 activity within Aroclor-induced mullet when compared with the noninduced fish. The high-pressure liquid chromatography analysis indicated that the major metabolites in the mullet induced with Aroclor 1254

ABBREVIATIONS: MFO = mixed function oxidase; BP = benzo[*a*]pyrene; OH = hydroxy; MCA = 3-methylcholanthrene; 4,5-, 7,8-, or 9,10-dihydrodiol = 4,5-, 7,8-, or 9,10-dihydroxy-4,5 (7,8-, or 9,10-)dihydrobenzo[*a*]pyrene; BP-7,8-diol-9,10-epoxide = one or more of the 7,8-dihydroxy-9,10-epoxy-,8,9,10-tetrahydrobenzo[*a*]pyrenes.

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were the 4,5-dihydrodiol, 7,8-dihydrodiol, and quinones of BP, whereas in the rat, the 9,10-diol was also produced in large amounts. The total amount of BP metabolites produced by the rat S-9 preparation was almost five times that of the mullet. This is in contrast to the observations of Pedersen et al. (*3*) and Ahokas and associates (*4*) who found much greater MFO activity in the noninduced trout as compared with the rat. In an in vitro system containing 25 μM BP, Pedersen et al. found that rainbow trout liver homogenate gave a specific activity of 2.404 μmol of 3-OH-BP/g protein per minute, whereas that of the rat was 0.167 μmol. In the comparison of BP metabolism in the trout (*Salmo trutta lacustris*) and rat that Ahokas and his co-workers (*4*) made, the substrate concentration was 25 μM and the temperature used was 37° C for the trout microsome mixture in the BP hydroxylase assay; the trout-to-rat specific activity ratio was 5.1.

Using the microsome fraction from the MCA-treated channel catfish, Hinton and co-workers (*11*) observed that the major metabolites produced at 30° C for 20 minutes in an in vitro system were the 3-OH- and 9-OH-BP compounds and the quinones. Significant quantities of the 4,5-, 7,8-, and 9,10-dihydrodiols of BP were also detected. All the metabolites except the 3-OH-BP were significantly increased in the MCA-treated S-9 preparations compared with the controls. The results of BP metabolism in rat and various fish systems are summarized in table 1. In all these experimental findings, incubation times of 15–20 minutes were used, so oxidase and epoxide hydratase action has been extensive. At the low concentrations of BP used in the flounder and salmon studies by Varanasi et al. (*14*), production of dihydrodiols has been considerably greater than in the systems in which the higher BP concentrations were used. Similar substrate concentration effects have been reported by Holder et al. (*16*) and Shen and associates (*17*) in the mouse and rat, respectively. From this summary of findings on the metabolite production, it appears that the level of enzyme activity in these fish is much lower than in the rat.

Most in vitro experiments conducted with rats as well as fish require an incubation time of 15–20 minutes. The amount of BP substrate for the MFO system has varied from less than 1 nmol to the most commonly used 100 nmol/ml per 1 to 2 mg of microsomal S-9 protein. With the Michaelis constant for the oxidase system in the range of 1 *M*, it is obvious that the MFO system probably had not been saturated with BP substrate in incubations in which the BP concentration was in the range of 1 *M* and 1 mg of protein, and the incubation times were 15 minutes or longer. Therefore, our studies have been on the effect of time on the metabolic conversions of BP in the mullet in vitro system (*18*).

When the incubation temperatures were 25° and 37° C, similar results were obtained, except that the optima for dihydrodiol formation occurred at 15 minutes at 37° C and 60 minutes at 25° C. James et al. (*19*) found that Florida fish had higher temperature optima for in vitro MFO activity than cold-water acclimated marine or fresh water

TABLE 1.—*Comparative metabolism of BP in the rat and fish*[a]

Animal	Treatment	BP						References
		3-OH	9-OH	4,5-Diol	7,8-Diol	9,10-Diol	Quinones	
Rat[b]	Control	272	29	37	10	16	117	(*12*)
	MCA induced	956	140	132	198	404	307	
Mullet[b]	Control	2	4	<1	<1	2	10	(*10*)
	Aroclor 1254	12	8	30	54	10	47	
Rat[b]	Aroclor 1254	—	76	112	267	166	157	(*10*)
Trout[b]	None	96	6	<3	80	64	40	(*13*)
Rat[b]	Control	95	<3	13	20	39	41	(*13*)
	MCA induced	574	51	56	206	262	168	
Flounder[c]	Control	270	Trace	—	460	64	Trace	(*14*)
	MCA	270	50	—	400	1,230	90	
Rat[b]	MCA induced	270	240	—	190	340	210	″
Salmon[c]	Control	100	40	—	280	222	70	″
	MCA	210	100	—	180	670	70	
Catfish[d]	Control	13.77	14.50	3.77	7.47	1.97	12.50	(*11*)
	MCA	13.60	19.20	6.03	12.13	3.77	16.40	
Little skate[d]	Control	49	14	4	14	8	4	(*15*)
	Dibenzanthracene	33	17	6	13	11	26	
Trout[d]	None	2.04 (phenols)		2.12 (diols)			0.44 (quinones)	(*4*)
Rat[e]	None	0.38 (phenols)		0.25 (diols)			0.35 (quinones)	(*4*)

[a] Dashes indicate no data were obtained.

[b] Specific activities are expressed as picomoles/minute per milligram protein (initial concentration of BP was 100 nmol).

[c] Values are expressed in picomoles per incubation of 2.8 ml of total volumes which contained 5 nmol BP.

[d] Values are in percent of total radioactivity eluted before parent BP (100 nmol BP was the initial concentration, 0.1 mg microsomal protein, and 20 min incubation at 30° C).

[e] Values are expressed as nanomoles of metabolites per 15-min incubation per milligram microsomal protein (100 nmol BP was the initial concentration with incubation at 30° and 37° C for trout and rats, respectively).

fish, findings that agree with our results obtained with the mullet.

When the metabolite formation in the mullet was studied in relationship to time of incubation by Melius and Tan (*18*), the production of dihydrodiols and phenols was linear with time to about 10 minutes at 37° C. An optimum production of diols occurred before 20 minutes, whereas maximum amounts of phenols were produced at 80–90 minutes. In contrast to the other 2 dihydrodiols, the 7,8-dihydrodiol was produced in increased amounts after 80 minutes; however, at 60 minutes it was hardly detectable. The phenols also were produced in significant amounts at about 15 minutes, but, at 25 minutes, small amounts were detected and then at 80–90 minutes the maximum amounts were detected. A maximum amount of quinone production occurred at 15 minutes. These results indicate the complex metabolic pathway for BP in the mullet microsome system, in which recycling of metabolites is probably taking place.

Following similar positive results reported in several previous studies (*2–5*), we confirmed the ability of fish to convert BP to mutagenic metabolites with the use of the Ames test (*7*). Mullet (*M. cephalus*), sea catfish (*Arius felis*), and Gulf killifish (*Fundulus grandis*) were induced with Aroclor 1254, MCA, and BP and MCA, respectively. The fish were acclimated in flow-through tanks (~250 liters seawater/hr, 18° to 22° C). After about 7 days, the appropriate dose of inducer in sterile corn oil was injected ip into the fish. The mullet were not fed during the 11-day induction period on the assumption that they subsisted on plankton. At 200 mg Aroclor 1254/kg body weight of mullet, the *Salmonella typhimurium* TA 98 revertancy rate by S-9 preparation from the mullet was five times greater than in the noninduced mullet S-9 preparation. The sea catfish were induced with 10, 20, and 40 mg MCA/kg body weight, and a linear dose–response relationship was obtained with a threefold increase in the revertancy rate of the *S. typhimurium* TA 98 over the control noninduced fish. The BP at a 30-mg/kg body weight level did not induce any increase in mutagenicity of the S-9 preparation of the killifish, whereas MCA at the same dose level caused a fourfold increase in the mutagenicity rate of TA 98. When mullet were induced with 200 mg Aroclor/kg body weight, the revertancy rate was 130, compared with 900 for the rat and 30 for the controls (*20*). When the killifish were induced with 30 mg MCA, the revertancy rate was 128 and for catfish, at a 40-mg dose, the revertancy rate was 117 (*20*). Hinton et al. (*11*) determined in their studies on the channel catfish (*Ictalurus punctatus*) that S-9 fractions from these fish exerted more of a mutagenic effect when incubated with BP and the TA 98 and TA 100 strains of bacteria than with the TA 1535 and TA 1537 strains. Thus it is now well-established that both fresh water and marine fish can metabolize the model polyaromatic hydrocarbon BP to mutagenic metabolites.

The known mutagenic BP metabolites (i.e., epoxides and dihydrodiol-epoxides) generated in the in vitro systems used in metabolism studies are highly reactive compounds: They react nonspecifically with proteins and nucleic acids, and they react with water to form phenols or are converted enzymatically to dihydroxydihydro derivatives. It is extremely difficult for one to detect the 7,8-dihydrodiol, 9,10-epoxide of BP directly by analytical methods, such as high-pressure liquid chromatography. However, the Ames test can act as a biological assay in the detection of mutagens. When BP is incubated with *Salmonella* mutants in this test with an Aroclor-induced S-9 preparation and an increase in the number of revertant bacteria is observed, probably a BP epoxide has been formed. It is also probable that the fish systems and the trout and mullet in particular produce the ultimate carcinogen and potent mutagen, the BP-7,8-diol-9,10-epoxide, as is suggested by the positive Ames test (*12, 20–22*).

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DISCUSSION

J. Schultz: When you speak of the rat and the mullet, how many individuals of each do you run, and what kind of comparison do you have among individuals? Is there a lot of variation?

P. Melius: Yes, there is. You will usually use 5 to 7 animals.

Schultz: Do you find the same kinds of variations in the rat? What strain of rat do you use? Is it a Sprague-Dawley?

Melius: Yes, Sprague-Dawley.

Schultz: Do they vary considerably also?

Melius: Yes, they do; but, of course, not as much as the fish.

Schultz: Do you have any kind of percentage variation in mind?

Melius: I do not have any numbers that I could give you, but there is a considerable amount of difference in the fish because we are using feral fish. Wide variations were obtained with the Ames test.

L. Poirier: Are the species of fish which produced the most diols also the most sensitive to carcinogenicity by the BP?

Melius: We have no information on that.

Benzo[a]pyrene Metabolism in Marine Fish and Some Analytical Aspects of Its Metabolites[1, 2]

W. Peter Schoor[3, 4]

ABSTRACT—In interspecies comparisons of benzo[*a*]pyrene metabolism, standardized procedures in the analysis of the metabolites must be established. Technical problems arising in metabolite analysis are discussed, and data are presented showing the activities of the mixed function oxygenase and transferase systems in phenobarbital- and 3-methylcholanthrene-induced mullet and killifish. — Natl Cancer Inst Monogr 65: 391–396, 1984.

The title of this Symposium, "Use of Small Fish Species in Carcinogenicity Testing," presumably allocates a role to biochemical investigation, the degree of which depends on the questions for which answers are sought. Observation of neoplastic changes at the organismic and cellular levels after chemical insult would be the most direct and reliable indicator of carcinogenesis. Intermediate in scope is bacterial mutagenicity testing, which, in the presence of a causative agent, may show direct mutagenic activity. Should metabolic activation of this agent be necessary for activity, the test can be performed in the presence of an isolated MFO system of any chosen species. This would be followed by biochemical investigations of metabolic changes at the subcellular level. Because no link between biochemical lesions and cancerous growth can currently be made, biochemical indicators cannot stand by themselves. However, were one to predict occurrence of cancer by studying proliferative changes only, the complex biochemical-pharmacological interactions occurring before the onset of cancer would be ignored.

This point, then, represents the intersect between the disciplines of pathobiology and biochemistry and is the starting point for the search for biochemical indicators. Thus biochemistry serves to support the pathological findings until the biochemical target site of cell proliferation has been found. There are signs that this site has been located, but the full circle from cause to effect has not been completed. If one assumes for the sake of argument that this site were known, the complete metabolic fate of a cancer-causing agent will still have to be elucidated to estimate how much of it must be at the outside of the cell membrane to yield a certain amount at the target sites. Because alternative pathways for most biochemical reactions (activation or deactivation) exist, their influence will greatly affect the final concentration at the target site and their effects must be known. Biochemically speaking, most metabolic pathways are known; however, their quantitative and interactive aspects are not as well understood. Investigators performing biochemical studies often ignore the cell wall and membranous structures, assuming total availability within the cell. However, most "free" substances in a cell are not free at all but are bound to varying degrees and thus affect internal regulation. The knowledge of an ultimate target site and the molecular species involved would be much more useful if all events which lead to this state were understood. An understanding of this phenomenon would also open the door to pharmacological manipulations enhancing one pathway over another and possibly affecting the ultimate carcinogenic complex. It is within these realms that biochemical and pharmacological experimentation are needed.

To find biochemical indicators of the onset of cellular proliferation, one must show that the active form of the causative agent can be composed in the tissue in question. The primary pathway by which water-insoluble substances are altered metabolically is mediated by the MFO system located in the MSF of certain cells. This fraction of cell membranes is usually isolated by differential centrifugation under widely ranging conditions, such as pH, buffer type, centrifugation time, gradient material, ratio of tissue weight to homogenization volume, and the number of resuspensions of the final preparation (the last condition is of great importance because of the removal of soluble proteins). This procedure needs to be standardized. Following isolation, storage of the MFO should be at −80° C and not extend without activity check past 30 days; once thawed, it should not be refrozen. The final MFO preparation yields a membrane suspension, the protein concentration of which is established with difficulty. Bovine serum albumin, a soluble protein, is usually used as a standard in the

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ABBREVIATIONS: MFO = mixed function oxidase; MSF = microsomal fraction(s); BP = benzo[*a*]pyrene; HPLC = high-performance liquid chromatography; 3-OH-, 5-OH-, or 9-OH-BP = hydroxy metabolites of BP; RFY = relative fluorescence yield; A = ampere(s); MCA = 3-methylcholanthrene.

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[2] Supported by the National Cancer Institute/Environmental Protection Agency Collaborative Program on Environmental Carcinogenesis, Project 3, Effects of Carcinogens, Mutagens, and Teratogens on Non-human Species (Aquatic Animals).

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[4] The author thanks Drs. D. R. Strength and S. Meera for their help.

measurement of insoluble membrane fragments, a practice which will not give accurate results. Inasmuch as we have few alternatives, a single method should be used to achieve comparable activities.

Additional complications arise during the final in vitro reaction of the MFO sample with a substrate. Wide variations occur in the uses of 1) concentration of co-factor (NADPH), 2) temperature and length of incubation, 3) introduction of water-insoluble test compounds into the reaction mixture, and 4) extraction and analysis of metabolites. All these can be standardized without much difficulty, and adjustments can be made when different animal species are used to yield optimal results. The analysis of metabolites, especially those of BP, has presented problems because the variety of analytical methods makes comparisons exceedingly difficult. Enzymatic activities have been based on "total extractable fluorescence" (*1*), disappearance of BP, and appearance of certain BP metabolites (*2*). The former type of analysis should be used only in a comparative sense because of its nonspecific nature and because fluorescence quenching and solvent effects are not defined. The measurement of the disappearance of the parent compound usually presents no great problem when reasonable care is taken, and results from different investigators can be compared. The most useful of all measurements without a doubt is the quantitative determination of all metabolites, particularly when other competing enzymatic reactions (conjugation) are done simultaneously with the oxidation. Total metabolite analysis is not as easy as it may appear, though cautious experimentation with an understanding of the underlying principles should give the desired results. Of greatest importance is that the analytical procedures be standardized so that the results of investigators can be compared.

Last but not least is the question of MFO induction in the laboratory situation. That MFO induction occurs in fishes and is caused by many of the same agents as in mammals is accepted by most working in this area of research. However, dosages and especially response times may differ among species. Administration of selected inducers is usually ip, im, or as an additive to food or water. The latter route would be the most preferable because it attempts to simulate field conditions, but facilities and initial trials favor the other methods. Any induction, either in the laboratory or field, should show changes in the metabolite pattern of a series of substrates. The work described here involves only 2 species of fish and only 1 carcinogen, BP; our intent was to find a fish species which could supplement the mammalian test species. The first choice was the mullet (*Mugil cephalus*) because it feeds by grazing on algae growing on rocks, skimming the surface of the water, and capturing any plankton of small size. When compared with the prey of a predator, this represents a much larger surface area of food to which highly adsorptive compounds are adsorbed. Mullet thus ingest toxicants such as BP adsorbed to the surface of its food. The major reason for our using the mullet as a test organism was the successful induction of the glucuronosyl and sulfate transferases (*3*). The MFO activity of the Gulf killifish is not as high as that of the mullet, and ip injection of an inducer is technically more difficult with the smaller fish. However, testing will continue with this and other small species.

MATERIALS AND METHODS

The 2 lobes of the mullet livers, dark red in color, represent from 1 to 2% of body weight, a convenient mass for the isolation of the MSF, the MFO activity of which has been described (*4*). Live capture and maintenance of mullet as test animals present difficulties. They are easily taken by gill netting, but removal from the net causes descaling and the animals usually die in a few days because of bacterial infection. The same problems exist with cast netting, even though our fish are collected in that fashion, and losses are high. Although we are able to keep survivors for up to 6 months during the cooler months, we know that they are nutritionally stressed (*4*). To induce the fish, we dissolved MCA and phenobarbital (acid form) in corn oil with the aid of sonification at 10-mg/ml concentrations to give solutions or suspensions to be administered to the fish at doses of 30 mg/inducer per kg of live weight. The first of 2 ip doses is given at day 0, the second on day 14, followed by termination at day 28. Fish were not fed but could obtain at least some food from the incoming seawater.

The MSF of livers were prepared by the method of Kinoshita et al. (*5*). The final MSF was resuspended in sucrose–Tris buffer and recentrifuged twice for removal of soluble proteins. Final preparations were in 0.05 *M* Tris, 0.25 *M* sucrose, pH 7.5, and stored at −80° C in small vials. Once thawed, contents were not refrozen. Determinations for MFO activity were made according to Selkirk et al. (*2*). Separation of reaction products was accomplished with HPLC. Identification and quantitation was based on the retention times and peak heights of mixtures of standard metabolites (National Cancer Institute) chromatographed under identical conditions. All solvents were of high purity suitable for liquid chromatography and were filtered through appropriate Millipore (0.25 μ) filters. Because the BP metabolite–DNA interaction is thought to present a critical step in the cause and effect of proliferative growth in mammalian systems, we performed preliminary physicochemical studies to see if such an interaction might also take place in fish. Highly polymerized salmon sperm DNA was dissolved in water at 0.1 mg/ml (sodium salt) and reacted with 9-OH-BP; then the RFY and changes in the spectrum of the 9-OH-BP were determined. The effects of methanol on the structure of the DNA and that of water on the fluorescence properties of the BP metabolite alone and in the DNA complex were carefully considered. We did another preliminary investigation in an attempt to establish whether complex formation with 9-OH-BP or an epoxide of BP would affect three-dimensional changes in the DNA. Inasmuch as we know that these changes can be followed with optical rotatory dispersion and circular dichroism, the spectra of the complexes were taken. The purity of all standard BP metabolites (National Cancer Institute repository) was checked by HPLC.

RESULTS AND DISCUSSION

In vitro activity determinations have been based mainly on the total metabolite evaluation or disappearance of BP,

TABLE 1.—*Partioning of 3-OH-BP from 1.0* N *sodium hydroxide*[a]

Phases	3-OH Species	Color	Polarity index[b]
Tetrahydrofuran	$\Phi\text{-}0^{-}$	Yellow	4.2
Aqueous sodium hydroxide	—	—	—
Hexane	—	—	0
Aqueous sodium hydroxide	$\Phi\text{-}0^{-}$	Yellow	—
Methyl ethyl ketone	$\Phi\text{-}0^{-}$	Yellow	4.5
Aqueous sodium hydroxide	$<10\%\ \Phi\text{-}0^{-}$	—	—
Ethyl acetate	$\Phi\text{-OH}$	—	4.2
Aqueous sodium hydroxide	—	—	—

[a] *See* text. Dashes = 0.
[b] Water = 9.

either of which can be misleading, and the results of the following experimentation must be taken into account when specific activities are resolved. The total metabolite evaluation is based on differential extraction with organic solvents from basic aqueous media. Fluorescence spectra of the phenols of BP differ greatly from those of their respective phenate anions, a property which one can use to follow extraction with certain solvents. With phenols, the pH of the reaction mixture as well as the solvent used for extraction must be carefully chosen for the analysis of the proper form of the compound. Table 1 shows a case in point when 3-OH-BP is partitioned from 1.0 *N* aqueous sodium hydroxide. The examples with tetrahydrofuran and ethyl acetate point clearly that, despite identical polarity indexes, the extracted forms of 3-OH-BP differ.

Were one to use fluorescence emission quantitation and a filter-type instrument, considerable error might occur (fig. 1). Further errors will occur if concentration-related quenching is not taken into account, e.g., 3,5- and 9-OH-BP isomers (table 2). Differences in the maxima of the RFY of these isomers are large, but they occur over practically the same concentration range. Another error can be introduced when methanol–water gradients are used during HPLC separations. Figure 2 shows the effect of water on the RFY of various phenols. The indicated values were obtained at concentrations that yield the maximum relative fluorescence, which, taken in methanol, served also as the 100% relative fluorescence points. Conditions for the diols and quinones have to be established.

Figure 3A shows the emission maxima wavelengths for the 12 isomeric phenol positions of BP. Positions 3 and 6 differ from the others, as they do when an RFY is assigned to the same positions (fig. 3B). Cautious interpretation supports free-radical action at the 6-position (*6*) and may explain high metabolite activity at the 3-position.

We made several attempts to gain insight into the DNA–metabolite interactions; the first was to use fluorescence to examine the physicochemical interaction that takes place. It was accomplished by our studying the change in the RFY. Table 3 shows that, at a weight ratio of approximately 1,000:1 of 9-OH:DNA under otherwise identical conditions, the RFY was much lower in the presence of DNA than in its absence. After 2 hours of UV irradiation, the RFY was reduced by about 5%. In the absence of DNA, under otherwise identical conditions, the RFY was reduced by more than 50% due to photolysis. Because the spectrum of the 9-OH was unchanged and little photolysis occurred, a charge-transfer type of interaction appears to have taken place. The second attempt focused on three-dimensional changes in the DNA molecule as a result of the complex formation shown above. The results revealed that no changes in the optical rotatory dispersion or in the circular dichroism spectra occurred at 25° C when salmon sperm DNA reacted with 9-OH-BP or the (*t*)-7,8-diol-9,10-epoxide of BP (fig. 4), which indicated no effect on the helix itself. Effects of higher temperatures are being studied.

In light of the above, metabolic studies were conducted

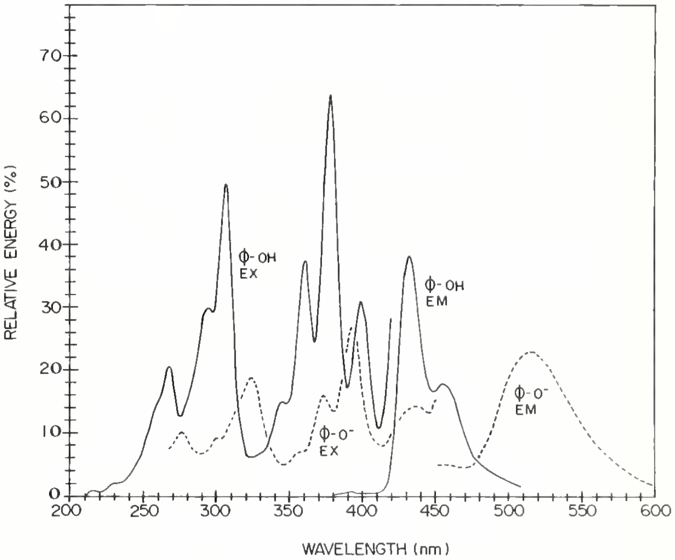

FIGURE 1.—Comparison of phenol and phenate spectra of 3-OH-BP. EX = excitation; EM = emission.

TABLE 2.—*Concentration-dependent fluorescence quenching in methanol*

BP metabolite	Concentration, ng/μl	RFY, μA/ng per μl
3-OH	0.666	11.3
	0.266	10.2
	0.133	12.9
	0.067	13.2
	0.033	13.0
	0.0067	9.0
5-OH	0.280	3.57
	0.112	4.10
	0.056	4.64
	0.042	4.52
	0.014	4.93
	0.0028	4.28
9-OH	17.0	11.1
	6.7	16.2
	0.40	14.8
	0.080	22.6
	0.040	22.0
	0.004	22.6
	0.002	20.5

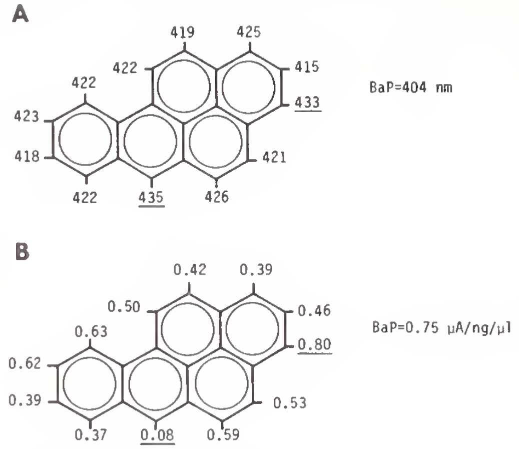

FIGURE 3.—A) Emission maxima (nm) in methanol of the isomeric phenol positions of BP (excitation at the highest wavelength, respectively). B) The RFY (μA/ng per μl) in methanol of the isomeric phenol positions of BP calculated from the emissions. BaP= BP.

with mullet induced with MCA. Earlier work (*4*) showed that induction required at least 14 days before MFO activity began to level off; later work (*3*) suggested that a second injection 14 days later and killing 14 days thereafter give more reproducible induction not only for the MFO activity but also for certain transferases. Following the analytical procedure of Elnenaey and Schoor (*7*), we determined MFO activity of MCA-induced mullet, sea catfish, and Gulf killifish. Using BP as substrate, we always found increased activity. Figure 5 illustrates the results of a 30-minute incubation with BP as substrate. Excellent separation was achieved with the newer instrument columns, with numerous metabolites shown, some of which await positive identification. The appearance of the 5-OH-BP among the metabolites of the MCA-induced mullet is unique and did not occur in the control animals, which indicated the possibility that at least for this species the appearance of 5-OH-type BP may signal induction by compounds like MCA. Of the quinones, only the 4,5-dione was positively identified when UV and fluorescence HPLC detectors were used. Compared with the other metabolites of BP, their UV extinction coefficients are high enough so that they can be seen easily by UV. They are highly unstable, especially in light, and must be purified before use as standards. Two groups of Gulf killifish were given ip injections of phenobarbital at rates of 12 and 25 mg/kg every 2 days for 12 days and then were killed after 14 days. Combined liver (5 fish) to body weight ratios of the fish were 2.2 and 1.4% for the 12- and 25-mg phenobarbital/kg doses and 1.0% for the controls. The MFO activities of the livers of the fish given the 12-mg/kg dose was significantly increased over those of the controls, although their activities were not as high as in the MCA-induced fish. No clear-cut induction was observed at the 25-mg/kg dose; perhaps toxic effects occurred at that level. Uridine-5′-diphosphoglucuronic acid-glucuronosyl transferase and sulfate transferase activities were also increased significantly over normal (*3*). When killifish were exposed

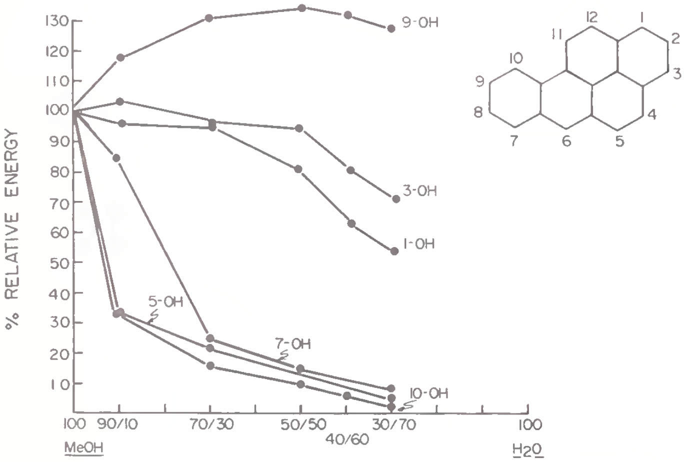

FIGURE 2.—Effects of water on fluorescence of some BP phenols.

TABLE 3.—*RFY of 9-OH-BP in various mixtures of water, methanol, and salmon sperm DNA*[a]

Amounts of mixtures		RFY, μA/ng per μl
5 μl 9-OH-BP[b]	+ 195 μl methanol	13.2
5 μl 9-OH-BP[b]	+ 195 μl water	1.94
5 μl Methanol	+ 100 μl water 95 μl DNA (0.34 mg/ml)	0
5 μl 9-OH-BP[b]	+ 100 μl water 95 μl DNA (0.34 mg/ml)	0.26

[a] Measurement was made at 378 nm (422 nm emission).

[b] Amount of 9-OH-BP=0.398 μg/μl.

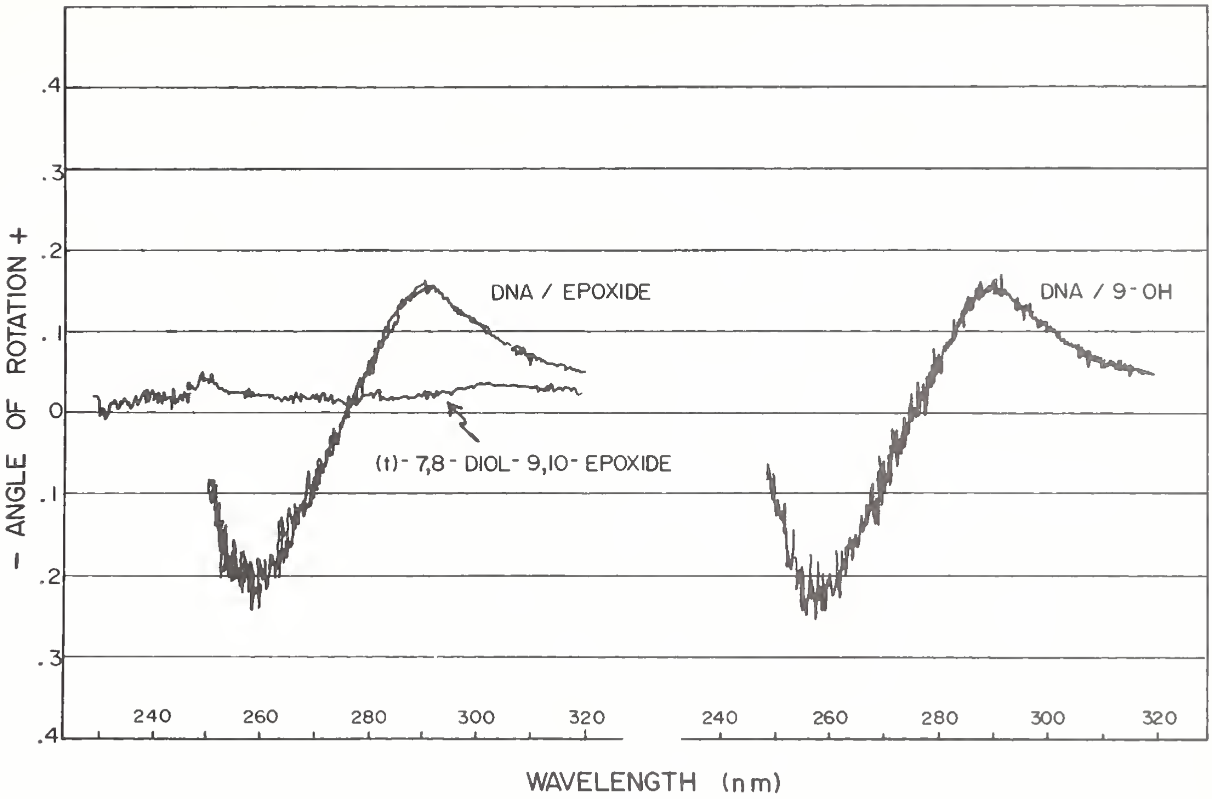

FIGURE 4.—Optical rotatory dispersion spectra of salmon sperm DNA reacted with A) 9-OH-BaP, and B) (*t*)-7,8-diol-9,10-epoxide of BP. BaP = BP.

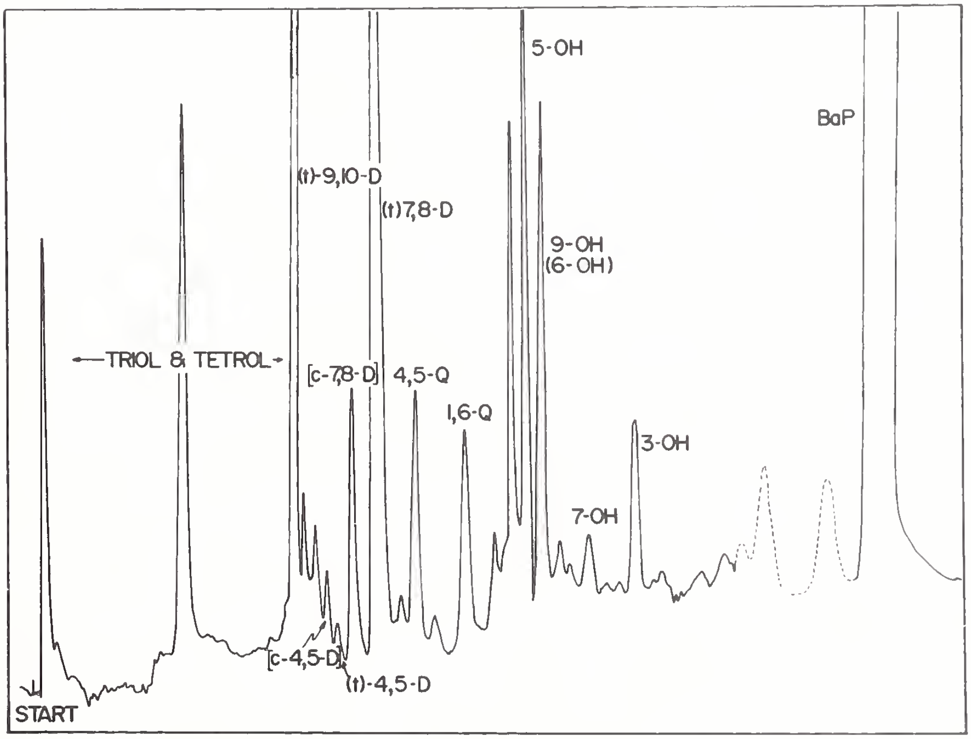

FIGURE 5. — Metabolites from MCA-induced mullet, 30 min MFO activity, with BP (BaP) as substrate.

to 0.3 and 0.03 mg phenobarbital/liter in flow-through exposures for 30 days, the percentages of liver to body weight of 8 fish averaged 2.1 and 1.9%, respectively, and MFO activities were slightly higher than those of controls.

SUMMARY

Few investigators doubt that fish are capable of metabolizing BP in much the same fashion as are mammals. Induction of MFO takes place, and metabolites are conjugated for excretion; care must be taken with the analytical biochemistry involved in their determination.

If species differences are to be discerned, individual metabolite patterns and conjugate formation must be established under various induction schemes. Therefore, investigators should standardize the analytical biochemistry as follows: induction as described here, incubation as in (*2*), MSF as in (*5*), and BP metabolite analysis as in (*2, 7*), and conjugation reactions according to Strength et al. (Strength DR, Daron HH, Schoor WP: Unpublished observations). This type of standardization would allow comparisons of their respective findings.

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Summary and Recommendations: A Consensus Report[1, 2]

Consensus Committee[3]

On Friday, December 11, 1981, thirteen participants in the Symposium met to discuss the future research needs of the field, management problems related to husbandry and testing techniques, and the overall potential of the use of fish in carcinogen testing. Initially, the group decided that these recommendations would address what Committee members perceived as important scientific gaps in the field. Discussion was limited to the following questions and will be presented in this report according to these general areas: 1) What future studies are recommended for investigators to assess and develop further the potential of fish as bioassay animals? 2) What criteria should be recommended in the selection of a fish species for development as a test animal in carcinogenicity bioassays? 3) What experimental procedures and conditions can be recommended in the design of a test protocol? 4) What are the chief advantages and disadvantages to be considered in the assessment of the usefulness of fish as bioassay animals in carcinogenicity testing?

FUTURE DIRECTIONS

Research on the responses of fish to carcinogens should encompass two broad investigative approaches: the study of these responses in controlled laboratory situations and the assessment of tumor induction in feral fish from polluted habitats. Recommendations for studies on chronic exposure focused on further investigations of the relative sensitivity of various fish species at different stages of development to a broader range of chemical carcinogens than those that have been tested in fish to date. The Committee believes in-depth studies to define specific physiologic, metabolic, genetic, and pathologic effects of carcinogen exposure on fish at the cellular or subcellular levels, or both, are essential. Specific recommendations concerning these areas are discussed.

Table 1 lists the compounds shown to date to be carcinogenic in fish. It illustrates both the preponderance of nitrosamines and aflatoxin B_1 and its metabolites used in such studies and the need for further testing of compounds representing all organic chemical classes and including those known to be carcinogenic or those with unknown but predicted tumorigenic activity. [With the exception of Hatanaka et al. (*10*), no one has assessed the response in fish to many different compounds of questionable or weak carcinogenicity in mammals.] In this way, the comparative sensitivity of fish to the carcinogenic effects of both known and suspect mammalian carcinogens can best be assessed. Therefore, the Committee recommended additional studies of this nature.

Secondly, dose-response data are crucial in 1) providing good comparative information on the behavior (toxic and carcinogenic) of a compound in fish versus higher animals and concomittantly in establishing those levels which are toxic to a fish prior to chronic exposure to the compound in the laboratory and 2) predicting the effects of a specific compound on feral fish. In considering the first reason, participants emphasized that the successful outcome of a chronic carcinogenicity investigation in their experience is heavily dependent on the preliminary range-finding toxicity study, a practice not always followed in previous studies with fish. Furthermore, Dr. Schultz noted the importance of the age of test fish as well. He found that the dosage of 7,12-dimethylbenz[*a*]anthracene which was finally used to induce tumors in *Poeciliopsis* in his laboratory was tolerated only by older fish. Thus the most suitable age as well as dosage levels to be used in the chronic test must be established in these preliminary range-finding assays. In predicting the effect of a chemical on feral fish (the second reason listed above), one must also examine a broad range of doses in the laboratory because exposure to a compound varies so greatly in the environment depending on its initial concentration, solubility, stability, turbidity, affinity for substrates, and biomagnification within the food chain.

To complement dose-response data and provide greater understanding of the biological differences found, the Committee also recommended investigation of the uptake, storage, metabolism, and excretion (i.e., the pharmacokinetics) of tested compounds. Additional studies involving identification of specific metabolites and their interaction with cellular macromolecules were also recommended. Dr. Jerry Hendricks noted that in-depth studies of this nature would best provide ideas for the direction of future research that would assess small fish for uses in carcinogenicity

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[1] Submitted for the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8-10, 1981.

[2] The comments that appear in brackets in this summary relate to background information which has been added by the scientific editor to clarify particular points introduced by members of the Committee.

[3] Participants included John A. Couch, Clyde J. Dawe, Harry K. Dupree, John B. Gratzek, Anthony Guarino, John C. Harshbarger, Jerry D. Hendricks, Karen L. Hoover, Takatoshi Ishikawa, Ikuo Kimura, Andrew D. Kligerman, John J. Lech, and R. Jack Schultz.

TABLE 1.—*Development of chemically induced tumors in various fish species*

Compound	Exposure route	Species	Target organ(s)	Earliest time to tumor development, wk	References
Aflatoxin B_1	Food	Guppy	Liver	36	(*1*)
N-2-Fluorenylacetamine	″	″	″	44	″
Dimethylnitrosamine	″	″	″	52	″
Sterigmatocystin	″	″	″	8	(*2*)
N-2-Fluorenylacetamine	″	″	″	32	(*3*)
o-Aminoazotoluene	″	″	″	12	″
4-Dimethylaminoazobenzene	″	″	″	16	″
Diethylnitrosamine	Water	″	″	7	″
Dimethylnitrosamine	″	″	″	10	″
Nitrosomorpholine	″	″	″	27	″
Dimethylnitrosamine	″	″	″	5	(*4*)
Diethylnitrosamine	″	″	Liver, esophagus	6	″
Nitrosomorpholine	″	″	″ ″	″	″
N-Dinitrosopiperazine	″	″	″ , GI tract	17	(*5*)
Diethylnitrosamine	″	″	″ ″ ″	7	″
Methylazoxymethanol acetate	″	Medaka	Liver	8	(*6*)
Diethylnitrosamine	″	″	″	″	(*7*)
″	″	″		5	(*8*)
″	″	″	″	12^a	(*9*)
Alfatoxin B_1	Food	″	″	NR^b	(*10*)
″ G_1	″	″	″	″	″
Sterigmatocystin	″	″	″	″	″
o-Aminoazotoluene	″	″	″	″	″
Methylazoxymethanol acetate	Water	″	″	″	″
Diethylnitrosamine	″	″	″	″	″
Diethynitrosamine	″	*Poeciliopsis*	Liver, hematopoietic tissue	28	(*11*)
7,12-Dimethylbenz[*a*]anthracene	″	″	Liver	″	(*12*)
Diethylnitrosamine	″	*Rivulus*	″	52	(*13*)
″	″	Zebra fish	″	10	(*14*)
Cycasin	Food, water	″ ″	″	NR^c	(*15*)
Nitrosomorpholine	Water	″ ″	Liver, intestine	10	(*3*)
″	″	″ ″	″ ″	7	(*4*)
Diethylnitrosamine	″	″ ″	″ , esophagus	8	″
Dimethylnitrosamine	″	″ ″	″ ″	5	″
Diethylnitrosamine	″	″ ″	Liver	13	(*16*)
Dimethylnitrosamine	″	″ ″	″	22	″
Aflatoxin B_1	Food	Rainbow trout	″	24	(*17*)
″ ″	Water			24^a	(*18*)
″ G_1	Food	″ ″	″	52	(*19*)
″ ″	Water	″ ″	″	″	(*20*)
″ M_1	Food	″ ″	″	″	(*21*)
″ Q_1	″	″ ″	″	″	(*22*)
Aflatoxicol	″	″ ″	″	32	(*23*)
″	Water	″ ″	″	52^a	^{d}
Dimethylnitrosamine	Food	″ ″	″	52	(*24*)
″	Water	″ ″	″	52^a	^{d}
Diethylnitrosamine	Food	″ ″	″	36	″
″	Water	″ ″	″	36^a	″
″	″	″ ″	Liver, kidney, swim bladder, stomach	36^e	″
Cyclopropenoid fatty acids	Food	″ ″	Liver	24	(*25*)
″ ″ ″	″	″ ″	″	32	(*22*)
N-Methyl-*N′*-nitro-*N*-nitrosoguanidine	Water	″ ″	Liver, kidney, swim bladder, stomach	36^a	(*26*)
″	Food	″ ″	Stomach	78	^{d}
Sterigmatocystin	Water	″ ″	Liver	52^a	(*27*)
Versicolorin	″	″ ″	″	″^{a}	″
Nitrosopyrrolidine	″	″ ″	″	″^{a}	^{d}
2,6-Dimethylnitrosomorpholine	″	″ ″	Liver, swim bladder, stomach	36^a	″
″	Food	″ ″	Liver, swim bladder, stomach		″

TABLE 1.—*Development of chemically induced tumors in various fish species (continued)*

Compound	Exposure route	Species	Target organ(s)	Earliest time to tumor development, wk	References
Dichlorodiphenyltrichloroethane	Food	Rainbow trout	Liver	72	[d]
Benzo[*a*]pyrene	″	″ ″	″	52	″
″	ip	″ ″	″	72	″
Methylazoxymethanol acetate	Water	″ ″	Liver, kidney, swim bladder, stomach	24[a]	″
Nitrosomorpholine	″	″ ″	Liver	36[a]	″
1,2-Dibromoethane	Food	″ ″	Liver, stomach	78	″
N-Methyl-*N′*-nitro-*N*-nitrosoguanidine		″ ″	Liver, kidney, swim bladder, stomach, gonad	20[a]	(*28*)
Dimethylnitrosamine	Water	″ ″	Liver	″[a]	″
Nifurpirinol	″	″ ″	″	104	(*29*)

[a] Fish were exposed to the compound once as embryos.
[b] NR = not reported; all fish were examined at or by 24 wk.
[c] All fish were examined at or by 44 wk.
[d] Hendricks JD: Personal communication.
[e] All fish were exposed 4 wk as fry.

testing.[4] The Committee members believe that the pooling of biologic material (such as livers for enzyme or in vitro hepatocyte suspension work) would provide a means of working with smaller species in which much less biologic material is available (compared with trout).

Members favored the investigation of a wide range of species, as was represented at the Symposium, rather than focusing on 1 or 2 presently promising candidates, such as the medaka and *Rivulus.* The Committee noted the importance of undertaking such studies utilizing new species in laboratories with adequate facilities and staff for the task. [Important requirements for such a laboratory would include the numbers of tanks that can be maintained, the ability to assay and dispose of contaminated water, the availability of breeding cultures, and the expertise of the staff in matters of husbandry for the particular species used. Also highly desirable would be the capability for histological processing of the fish and pathologic assessment of their tissues (these could be performed through collaborative or contractual agreements with another laboratory) and, preferably, on-site evaluation of physiologic parameters, e.g., blood and liver enzyme chemistry.] Such broad-ranging investigations of various species might not only uncover new promising species to be used in laboratory testing but may also prove useful in future studies designed to define biological and biochemical differences between susceptible and resistant fish species. This is an area that members concurred had considerable potential because the development of genetically identical fish is currently possible: The availability of *Rivulus* and *Poeciliopsis* sp. and the Amazon molly as well as artificial parthenogenetic techniques (as described by Streisinger) will enable investigators to examine the comparative response of a carcinogen against different genotypic backgrounds within the same fish strain as well as responses to various carcinogens in one defined genotype. This area of research can yield not only an enhanced understanding of the mechanisms of carcinogenicity in one compound but can help establish a particularly sensitive genotype for testing purposes. Dr. Jack Schultz noted another potential use by establishing effective carcinogenic levels of a compound in homozygous or isogenic fish maintained under defined conditions in the laboratory; comparison of these levels with concentrations that are effective in the same fish maintained under one or more simulated environmental conditions (e.g., addition of a known water pollutant to aquarium water) could indicate the influence of environmental factors on tumor induction or rate of tumor development in the fish. Moreover, Dr. Schultz emphasized the need to develop genotypes representative of feral animals to gauge the effect of naturally selected metabolic pathways which might detoxify rather than produce carcinogenic metabolites, thus making fish in the wild less susceptible to some water-borne carcinogens. He pointed out the lack of such genotypes in rodent test strains.

In addition to exposure of completely developed fish to a compound, the Committee discussed the strong potential for the egg embryo exposure technique. In further developing the usefulness of this technique as a screening method in carcinogenicity testing, the Committee discussed the problem of introducing water-insoluble compounds into the fertilized egg. Because of the impermeability of the chorionic membrane to such substances, the means of exposure is limited effectively to water-soluble compounds and those which can be introduced by a carrier. Alternative methods need to be explored, such as the use of a microinjector to introduce the substance through the chorionic membrane. Dr. Clyde Dawe also suggested the direct injection of a compound into the heart of the developing embryo because this organ is readily visualized.

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[4] At Oregon State University, studies designed to define the high or unusual sensitivity of rainbow trout to aflatoxin B_1 have been and are currently being conducted, including the assessment of total body burden to various carcinogens, biochemical characterization of the handling of aflatoxin B_1, and metabolite interaction with DNA. These latter studies are supported by in vitro hepatocyte culture work as well. The in-depth study at Oregon State of aflatoxin B_1 metabolism exclusively within the highly sensitive rainbow trout led to the important and previously unreported observations (in any other species) that the metabolites versicolorin A and aflatoxin Q_1 were carcinogenic.

Another area which requires strong emphasis is the use of in vitro work, primarily as supportive research for mechanistic studies. [This would include the establishment of viable primary and established hepatocyte cultures for enzyme and metabolism work as well as a more rapid means for exploration of the effect of chemical promoters and initiators on fish cells.]

In field studies, the Committee suggested the increased use of serum analyses on feral fish for assessment of the effects of water pollution; possible factors would include levels of pollutant metabolites or oxidative enzymes, or both. [Other endogenous enzymes, such as serum glutamic-oxaloacetic transaminase for liver damage could be examined as well.] Better advantage might also be taken of routine surveys conducted by state Departments of Natural Resources or environmental agencies on feral fish in polluted rivers such as the Hudson and the Ohio. For example, Dr. Harshbarger cited his collaborative work with the Ohio River Sanitation Commission, a survey which is conducted annually through the joint efforts of Ohio, West Virginia, and Pennsylvania. Many of these surveys do not include gross and histopathological examination of the fish specifically for tumors, but such opportunities with a population of fish could certainly prove most useful. [This has been borne out in surveys such as those undertaken along the Hudson and Black Rivers and in Puget Sound; hepatocellular tumors were found in fish living in both sites.] Dr. Harshbarger suggested that specifically polluted river sites ought to be targeted for sampling by state Departments of Natural Resources in collaboration with the Registry of Tumors in Lower Animals to ensure a standardized survey method for fish tumor prevalence, uniform tissue processing, consistent evaluation of data, and easily accessible central data storage.

Lastly, the Committee brought up the need for increased standardization of histological lesions in fish; this is an area which, particularly with liver lesions, is dependent on the known pathogenesis and histological typing of mammalian lesions. A consensus on toxic, preneoplastic, and neoplastic lesions, although not yet attained in rodent pathology, has not even been attempted among researchers interested in fish pathology and could be the basis of a most valuable meeting in the future.

CRITERIA FOR SPECIES SELECTION

Criteria for a suitable fish species for testing will, of course, differ according to which of two broad research areas applies to the proposed fish studies: These two directions are 1) laboratory investigations that comprise basic mechanistic studies as well as applied bioassay procedures and 2) field surveys in which the test species serves as an indicator organism of carcinogen contamination in the waterways. The following criteria were proposed:

Ability of Fish to Thrive on a Purified Diet

This capability is particularly important in a test species in which experimental variables must be minimal. A defined diet is clearly preferable to the feeding of live food (such as brine shrimp) which may contain halogenated hydrocarbon contaminants. Moreover, because the dietary needs of certain fish species are not well-defined, it is impossible for one to maintain such fish in the laboratory for several months. As an example, Dr. Kligerman cited his experience with the mudminnow which accept only live food. Therefore, it is critical that the dietary habits of the fish chosen for long-term laboratory studies be satisfactorily characterized. If, in the rearing of young fish, live food must be included in the diet, it should be analyzed for possible chemical contaminants.

Disease-free State

Achievement of a disease-free state is particularly difficult in fish cultures in which mycobacteria is a chronic problem and extremely difficult to eliminate. Fish stocks should be obtained from existing laboratory facilities in which particular cultures are customarily maintained and their disease-free status assured. Thus the investigator should obtain from the source the disease-related history of the species of choice as well as any safeguards used in disease prevention or treatment of the fish. An alternative is the establishment of a breeding colony within the laboratory, provided that sufficient staff members are familiar with the husbandry techniques involved in fish cultures. This may require a considerable committment in staff, time, and equipment and should only be considered if personnel are familiar with the essential husbandry techniques. As in the rodent systems, selection of genetically predisposed or resistant strains is a potentially fruitful area of research (as discussed above). Thus selective breeding or the artificial parthenogenesis techniques with eggs could lead to the development of special fish strains eminently suitable for the investigation of responsiveness to carcinogens.

Hardiness Within the Laboratory

Such features ideally would include the tolerance of water temperatures within a 5° C range and the ability of the fish to withstand some variation in water chemistry (pH, nitrite levels, and hardness).

Continuous Accessibility of Fish

An assured supply of fish throughout the year (either within the testing laboratory or by the original supplier) is highly desirable and may well be critical to the success of a bioassay; such accessibility is only possible if optimal conditions for spawning are known and can be achieved in the culture system. Again, personnel expertise in husbandry techniques, including breeding conditions, either at the supply source or within the investigator's laboratory is essential.

Size

Ideally, a larger fish, e.g., the sheepshead minnow or killifish, would be particularly favored as a test animal if biochemical characterization of serum, liver enzymes, etc., is to be done. However, among small fish species, it is the extremely small fish such as the medaka and guppy that to date have proved to be most sensitive. The committee suggested pooling tissues (e.g., the liver) in such instances. However, this is an extremely tedious procedure, and extensive biochemical studies might best be done in trout or await those investigations that may describe larger fish in which tumors can be chemically induced. [With increasingly sensitive separatory techniques, i.e., high-performance liquid chromatography, progressively smaller amounts of tissue are required for one to obtain metabolite patterns. Other procedures that require radioimmunoassay and monoclonal antibodies may also prove extremely valuable in solving this problem.]

FIELD STUDIES

Field studies can be conducted either by routine epidemiological surveys of a particular fish population within the waterways being monitored or by introduction of a test species confined within cages into the water to be monitored. In the latter type, short-term exposures with genotoxicity as an end point (e.g., measured by sister chromatid exchange) could be performed. Alternatively, long-term exposures with tumor induction as an end point could be studied. If possible, fish native to the area under study should be utilized. Such use would, of course, increase the probability for survival under existing water conditions. However, investigators find it highly desirable to work with fish in which tumors have been induced and more productive to choose species with a known sensitivity to some tested carcinogens or a documented capability to metabolize specific classes of carcinogens. A participant pointed out that there may well be a trend toward selection of genotypes among fish species that are more carcinogen resistant (by way of more efficient oxidative and conjugative metabolizing systems); thus field surveys of endogenous fish may be less revealing than is introduction of specific fish species into the aquatic site to be monitored.

Field studies may also encompass exposure of fish to contaminated water from a specific environmental source within the laboratory. Dr. Kligerman proposed that a battery of fish species (including larger species such as trout) would be desirable. Suggested species endogenous to the United States and some advantages for their use include:

Species	Advantages
Rainbow trout (*Salmo gairdneri*)	Freshwater fish Known sensitivity to wide range of carcinogens
Catfish (*Ictalurus* sp.)	Freshwater fish Subsistence possible on purified diet Ability to be reared in laboratory setting Widespread distribution in the United States Persistence in polluted habitats High prevalence of tumors in several bullhead populations from industrially polluted areas
Oyster toadfish (*Opsanus tau*)	Marine fish Few large chromosomes (useful for genotoxicity testing)
Killifish (*Fundulus grandis*)	Marine and brackish water fish Widespread distribution Ability to be reared in laboratory setting
Mudminnow (*Umbra* sp.)	Freshwater fish Few large chromosomes (useful for genotoxicity testing)

Valuable basic information and a determination of the most sensitive strain could be obtained by the testing of several genotypes within a fish species. This could easily be applied to field studies, probably to those in which fish are exposed to polluted water either in cages at the environmental site or within the laboratory with the water to be tested carried in and introduced into the aquaria.

PRACTICES FOR CARCINOGEN TESTING: EXPOSURE ROUTES AND TEST PROTOCOLS

Routes of Exposure

The chemical nature of the compound as well as the most common origin in the human environment (e.g., a pollutant in the water or an industrial product or by-product) should determine whether the exposure route will be by way of the water, incorporation into the diet, or (least preferably) by ip or iv injection. Ideally, water exposure is the most suitable because this route has been claimed to be the most effective in tumorigenesis studies by Khudoley, and test animals will thereby be constantly bathed in the carcinogen. Water is taken in through the gills in freshwater fish and drunk as well by marine fish. Dr. John Couch pointed out that static water exposure studies are extensively used at the Environmental Protection Agency Laboratory in Gulf Breeze, Florida. Water exposure has been the sole route in the highly successful work with carcinogen treatment of embryos as well. However, the compound must be able to penetrate the chorionic membrane either alone or by a carrier. If the compound is considered particularly hazardous to humans, is refractory to removal by charcoal or other types of treatment filters, and is insoluble in water, the embryo exposure procedure could be most suitable. Alternatively, the carcinogen could be added to the food or given by injection. Trout are a particularly suitable species for dietary exposure studies because they are avid consumers, according to Dr. Hendricks. Both ip and im injections are the least suitable means of introducing the compounds in small fish, particularly, because problems occur with an unknown portion of the injected material escaping from the site of injection, inherent trauma, and possible anesthetic shock.

In regard to dietary exposure, Dr. Harry Dupree mentioned that the problem of unpalatability could be solved by administering an increasingly heavy concentration of the compound that has been mixed with the food for a 2-week period. If given in this graded fashion, fish may more likely accept an otherwise unpalatable diet.

Test Protocol

In a short-term screening bioassay, which could be a part of a larger tier system, multiple species of fish could be examined. Thus the probability would be increased that if a compound can be metabolized to a carcinogenic metabolite, at least one of the species may be appropriate (through the mixed function oxidase system or detoxification pathways) to show a tumorigenic response. Secondly, if the compound were a contaminant of waterways, species which live optimally at high and low temperatures in saline or fresh water may be used to cover a range of aquatic conditions under which pollutant exposure may occur in nature. With compounds that normally are chemically unstable or scarce in purified form, the embryo exposure technique requiring an average exposure time of 1 hour provides an excellent alternate mode of testing and significantly reduces hazardous laboratory conditions and exposure of the fish at a particularly sensitive period in the life cycle. Although this system was developed and is extensively used in rainbow trout, Klaunig reported on its use in the medaka (*see* page 155). [One of the participants at the Symposium had expressed concern over the use of plastic culture plates in an exposure protocol. He suggested that the carcinogen used in this exposure could adsorb to the culture vessel wall and thus decrease the concentration of the carcinogen or chemicals within the plastic culture plate itself, such as plasticizers and catalysts, could leach out and subsequently act as promoters or co-carcinogens.]

In assessing the histological results, investigators might consider liver imprints stained with toluidine blue for a rapid estimation of tumor development in small fish species. The small size of some proposed fish species also allows use of parasagittal (dorsal to ventral aspect) or hemisagittal (lateral) sectioning of the total fish and thus provides a highly satisfactory assessment of effects in all tissues when they are sectioned.

Although small fish species can more easily be maintained in large numbers within a laboratory setting, such numbers are probably unnecessary (barring problems with unexpected disease outbreak, etc.) because the background incidence of tumors in those species which have been most studied is low.

CONCLUDING STATEMENTS

Though relatively young, carcinogenesis studies and field surveys with fish promise to be particularly fruitful if the interest and, more importantly, the funding, are available for future investigations. Papers presented at this Symposium illustrate this remarkable potential as well as the special problems related to carcinogenicity in fish. Both advantages and disadvantages must be acknowledged if we are to provide recommendations for the future course of this work. As presented by various investigators at the Symposium and summarized by members of the Consensus Committee, the most important problems relate to the limitations of extrapolating carcinogenicity results in a physiologically and anatomically different animal from man, the special dietary needs of some species, and the disposition and disposal of carcinogen-contaminated water in static and flow-through systems. The work of Kyono-Hamaguchi ascertained that the poikilothermic nature of fish can influence the response to carcinogens, most likely because of the different temperature optima for the action of specific mixed function oxidase or conjugative enzymes. Although the results of investigators conducting intensive enzyme research have demonstrated similar (though often lower) oxidative and conjugative metabolic pathways in the larger fish species, e.g., the trout and mullet, such studies have not been performed extensively with the smaller species. Tumor induction as a response to aromatic amines has not been seen in the rainbow trout in which *N*-2-fluorenylacetamide has been tested. Major anatomical and physiological differences including the lack of lungs and a different reproductive system pose questions as to the sensitivity of animals that do not possess homologous organs. The liver is the major and almost exclusive target tissue among carcinogen-treated fish. Unknown dietary requirements and sometimes the absolute need for supplemental live food makes the maintenance of such fish impossible on a more defined diet as in the standard protocol of rodent bioassays. This fact may add perplexing unknowns to the experimental conditions under which the test is run. Lastly, the problem of disposal of large quantities of carcinogen-contaminated water in both static and flow-through systems must be solved if continuous water exposure is to be undertaken. Moreover, the uptake of carcinogen by other elements in the system, including tank walls, gravel, filter tubing, and algae should be clearly ascertained and, consequently, the actual levels of carcinogen carefully followed and adjusted, when required, if wide fluctuations in the levels of compound to which the fish are exposed are to be avoided.

Apart from the lack of homologous target organs,[5] these problems are not insurmountable and, in regard to dietary needs and water disposal, reflect the lack of necessary research in a young field. Moreover, the poikolothermic nature of fish can conceivably be put to good use by establishment of the optimal temperature for hastened development and maximum frequency of tumors in experimental fish.

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[5] Although fish may not develop organs homologous to those of mammals, they do possess tissues which have a similar physiological function (analogous tissues); e.g., portions of the anterior kidney secrete adrenocortical hormones, and the ultimobranchial gland regulates calcium metabolism similar to the mammalian parathyroid gland.

In addition, several major advantages of fish argue for their continued development. These include 1) their excellent suitability as biological test specimens because of their continuous exposure to water-borne chemicals; 2) their hardiness and the ease of handling large numbers of numerous species of fish within the laboratory; 3) the ability of many small fish species to live at warmer temperatures, a condition which could hasten tumor development in the laboratory; 4) the low background incidence of tumors in fish; 5) the short latency already observed in some fish species; 6) the comparative ease in which all tissues in small aquarium fish can be assessed histologically by use of midsagittal or coronal serial sectioning; and 7) the induction of tumors with extremely small amounts of carcinogen in a limited time and space (egg embryo exposure technique). Lastly, fish may prove to be more acceptable animals for in vivo screening of carcinogens (from the standpoint of the guidelines for animal experimentation) than those proposed in vivo short-term tests utilizing Strain A mice or hamsters (*30*). Costs for such a short-term in vivo system are considerably lower than those required for a 6-month test in mammals. According to Dr. John Gratzek, costs for the daily maintenance of a fish are 30-fold lower than for mice and 60-fold lower than those for a rat. This does not take into account necropsy and histological costs, but, for small fish, most of the work would involve a sagittal cut through the fish with any gross observations made at that time, followed by subsequent step sections.

In conclusion, the Committee urged that the use of fish should be promoted most strongly in those areas in which they are most useful in testing, i.e., as indicators for carcinogenic activity of water-borne substances or for tests in which studies are impractical in other animals. This latter category would include the testing of compounds that are not available in large amounts (the egg embryo exposure technique) or are unstable at high (physiological) temperatures. Furthermore, an ultimate goal of the investigators who presented the results of their studies at this Symposium is to assess fish as appropriate test organisms for the initial testing of suspect carcinogens. The effort involved in the long-term testing of compounds in rodents is substantial, and a short-term in vivo bioassay in fish could be most useful in augmenting available information that researchers require to make the decision to initiate the long-term rodent assay. Lastly, the unique biological and physiological features of fish promise fruitful investigations into the nature of cancer and its chemical induction.

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